SEDIMENTATION AND ELECTROPHORETIC CHARACTERISTICS OF CRYSTALLINE GLOBULIN FROM CUCURBIT SEEDS

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The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled "Sedimentation and Electrophoretic Characteristics of Crystalline Globulin from Cucurbit Seeds" submitted by David Gene Anderson in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Various buffers, buffer concentrations, and pH values were used in both electrophoretic and sedimentation analyses to study their effects on dispersions of crystalline globulin from the seeds of four cucurbit species. The effects of age on dispersions were studied in the ultracentrifuge.

The cucurbit seed globulins differed electrophoretically with the main difference occurring at pH 4.8 and pH 4.3.

A decrease in mobility with a decrease in pH was observed below the isoelectric point, while a decrease in mobility with an increase in pH was observed above the isoelectric point.

Seed globulins from the four cucurbit species were shown to be labile association-dissociation systems. The age of the dispersions had a marked effect of association-dissociation phenomena.

A decrease in sedimentation coefficients and in association of the cucurbit seed globulins with a decrease in pH was found below the isoelectric point.

Dissociation was favoured at pH values away from the isoelectric point and in solutions of low salt concentration.

The seed globulins from the four different cucurbit species differed in their sedimenting characteristics under varying conditions of pH, salt concentration, and age of dispersion.

THE UNIVERSITY OF ALBERTA

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A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

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INTRODUCTION

Seed proteins have been of continuous interest to research workers. A wide variety of measurements, both physical and chemical, have been made in an attempt to clarify the nature of these biologically indispensable compounds. Much attention has been paid to methods of preparation and purification since these hold prominent and vital roles in protein investigation. Identification of the nature and number of molecular species has rested largely on amino acid studies, end group analyses, and physical measurements such as solubility, sedimentation characteristics, electrophoretic characteristics, and light-scattering phenomena.

Differences among similar proteins from various species of organisms have been noted in some cases and measurements on these dissimilarities are accumulating. The meager physical measurements made on proteins of the Cucurbitaceae family have failed to show specific differences but amino acid analyses have. In view of the differences found in amino acid composition, results from physical measurements such as sedimentation and electrophoresis should yield interesting results. Therefore, in this study the globulin from the seeds of squash, pumpkin, cucumber, and watermelon were studied using these two methods.

As early as 1883 Schulze and Barbieri, as referred to by Smith et al. (67), isolated phenylalanine from squash seed globulin hydrolysate. Reference is made to Osborne (23) who, as early as 1892, apparently prepared crystalline globulin from several genera of Cucurbitaceae.

Cucurbit seed globulins have been of practical interest as a possible substitute for hemp seed edestin. Since production of hemp is regulated by marijuana laws, the advantages of using material from a plant as common as the cucurbits are obvious.

The cucurbit globulins are of fundamental interest as the one from squash (23) has been shown to be an association-dissociation system. Haurowitz (27) states that reversibility of protein association-dissociation systems may be of biological importance because, if disaggregation occurred within the living cell, it would bring about an increase in osmotic pressure, a swelling of the cell, and a sudden drop in viscosity of cellular fluid. Information on the nature of the active binding sites in protein can be obtained by studying any association-dissociation phenomena that may exist.

This study was designed to find differences that exist among the crystalline globulins from the species squash (Cucurbita maxima), pumpkin (Cucurbita pepo), cucumber (Cucumis sativus), and watermelon (Citrullus vulgaris). It was also hoped to obtain information on any association-dissociation phenomena which may occur with these proteins.

LITERATURE REVIEW

Several authors refer to Osborne's work of 1892 (23, 45, 81) in which crystalline proteins were obtained from squash seed. Various procedures have been described since then for crystallizing globulins from a number of Cucurbitaceae species and varieties (31, 45, 52, 53, 79, 82).

A number of methods have been used to prepare globulin from cantaloupe (Cucumis melo) and squash (Cucurbita maxima). The

most satisfactory method appears to have been assumed to be extraction of the globulin from the hulled, ground seeds with 2% sodium chloride solution. Crystals of octahedral shape separated from the extract on cooling (79). Crystalline globulin was obtained from hulled, ether-extracted watermelon (Citrullus vulgaris) by cooling the 2% sodium chloride extract from 50°C (52). Globulin was precipitated from a 1.25% sodium chloride solution by cooling from 70°C. The globulin had been extracted from hulled, ground watermelon, gourd, and pumpkin seed with 5% sodium chloride (82).

Vickery, Smith, Hubbell, and Nolan (79) modified the methods of Osborne chiefly by omission of the fat extraction step, and by the introduction of a heat coagulation step designed to remove minor quantities of proteins, presumably of the albumin type, before precipitation of the globulin. They found it unnecessary to remove seed coats prior to extraction of the globulin. The 10% sodium chloride solution in which the globulin had been extracted was siphoned from under the fat layer, filtered, and the globulin precipitated by adding four volumes of distilled water and cooling. Recrystallization could be done as many times as desired by taking the crystals up in two-thirds the original volume of sodium chloride solution followed by dilution. Good crystals could be kept in the refrigerator under toluene for some time. Crystallization was successful with globulin from Cucurbita moschata, Cucurbita pepo, Cucurbita maxima, Citrullus vulgaris, Cucumis melo, and Cucumis sativus.

Fuerst (22) modified the method of Vickery et al. chiefly by carrying out fat extraction with ether and by omission of the heat coagulation step. Later, Fuerst, McCalla, and Colvin (23) used essentially the same method as Fuerst (22) on squash protein for sedimentation studies.

It seems that all extraction procedures of the cucurbit proteins have involved crystallization from sodium chloride extracts.

Although crystalline proteins provide a convenient and consistent starting material, they should not be thought of as being homogeneous. Heterogeneity has been demonstrated electrophoretically in crystalline pepsin (32). By electrophoresis, two components have been found in crystalline pepsin, crystalline trypsin, and crystalline lysozyme (5). The review by Colvin, Smith, and Cook (17) shows that the purity of so-called pure proteins, whether crystalline or not, must be regarded as relative and cites various examples of what is called microheterogeneity. It should be recognized, however, that in most cases the heterogeneity of crystalline proteins has been caused by association-dissociation phenomena. Thus, crystalline proteins usually consist of at least very closely related components.

Studies have shown oxaloacetic decarboxylase activity in the globulin from pumpkin and squash seeds (78). It is of interest to note that Byrrum, Brown, and Ball (14) found 0.12 mg. manganese per 100 gm. protein and 0.02 mg. iron per 100 gm. protein besides traces of zinc, cobalt, and nickel in recrystallized material. Their experiments indicated the possibility that oxaloacetic decarboxylase from cucurbit seed is a metal-protein. Hence it may follow that, in the presence of added metal ions, increased activity of the enzyme, isolated from other sources, results from the formation of a metal-protein complex. Such information may be a clue to the active binding centers on the cucurbit globulins. It may also be a clue as to the ions that would have a pronounced effect on these proteins.

Amino Acid Studies

The most extensive work on the cucurbit seed globulins was done after passage of the United States Marijuna Law of 1937. The attempt to find a substitute for edestin resulted in detailed studies of Cucurbitaceae. Amino acid analyses on some of the cucurbit seed globulins are complete (74). On a relatively large number of others, they are fairly comprehensive (65).

Smith and Green (65) have summarized the amino acid composition of pumpkin, squash, watermelon, and cucumber. They found no difference in amino acid content of squash and pumpkin that could not be explained by errors in analyses. Watermelon and cucumber can be distinguished from each other and from squash and pumpkin on the basis of amino acid content.

The minimum molecular weights as calculated from the amino acid composition of the globulins are: watermelon, 55,000; cucumber, 55,000; squash, 58,000; and pumpkin, 58,000 (65).

Thompson and Steward (74) used chromatographic methods to determine the amino acid content of cucumber and squash globulin using the same varieties as used by Smith and Green (65). They got no significant difference in valine content whereas Smith and Green did. A definite difference in phenylalanine content was found by Thompson and Steward (74) whereas Smith and Green did not find a difference.

It would be beyond the scope of this thesis to give a complete review of the amino acid findings. It is probably sufficient to note that they are in the literature (26, 28, 29, 30, 31, 45, 52, 61, 65, 66, 67, 69, 74, 79, 81).

Leontjew (53) carried out measurements on the seed globulins of watermelon (Citrullus vulgaris), melon (Cucumis melo), and squash* (Cucurbita maxima) and found them identical. The study consisted of specific rotation, colorimetric observation, crystallography, refractive indices of crystals, and a study of precipitin reaction. As the solvent was 0.5 N sodium hydroxide, derivatives of the globulins were probably studied.

Electrophoresis of Squash Globulin

The physical characteristics of cucurbit globulin molecules were practically unknown until Fuerst (22) carried out an electrophoretic study of dispersed squash seed globulin at various pH values. Byerrum, Brown, and Ball (14) carried out an electrophoretic study on squash seed globulin at pH 4.0 only and found it to be essentially homogeneous. Fuerst (22), however, experimented with squash seed globulin in 0.2 M sodium benzoate, pH 4.7; 0.05 M sodium formate, pH 3.7; 0.05 M sodium acetate, pH 3.9; and 0.05 M sodium acetate, pH 4.7. Only in the 0.05 M sodium acetate, pH 4.7 did the globulin appear as two distinct components. Unpublished results of work done by Fuerst at the University of Alberta show only one component in 0.1 M glycine NaOH buffer at pH 10.2, 10.7, and 11.4. In 0.1 M glycine at pH 9.8 there was an indication of two components. There appear

* The author uses the term "Kurbisse" for Cucurbita maxima and although German-English dictionaries translate "Kurbis" as pumpkin, it was thought that squash, contrary to the Chemical Abstracts, would be a better translation. Evidently "Kurbis" is a very general term.

to be two components on electrophoresis in 0.033 M borate buffer at pH 7.9, 8.3, 8.7, and 9.3. The ascending boundary separated into two distinct peaks but the descending boundary in some cases was only strongly skewed. This was the case for all the pH values from 7.9 to 9.7.

Fuerst et al. (23) got electrophoretic results inconsistent with sedimentation results.

As has been stated before, crystalline proteins are not necessarily homogeneous. Examples of this have already been given. Heterogeneity of some of the solutions of crystalline proteins has been found to depend on pH and ionic strength, among other things. Fuerst et al. (23) point out the danger of drawing conclusions from single electrophoretic determinations. Any worker reporting a single determination on protein has limited the interpretation of his work and has no assurrance that the number of components may not be quite different under different conditions.

Association-dissociation Phenomena in Protein Systems

The dissociation of protein molecules into smaller units is not unusual. It appears to be a phenomenon of considerable frequency. Svedberg (71) went so far as to say that all proteins were different complexes of the same size unit molecules. The basic unit supposedly had a molecular weight of about 17,600. This "Multiple Law Hypothesis" has been severely criticized by Johnston, Longuett-Higgins, and Ogston (44). The research to date does show that many proteins as they occur in living organisms are complexes of lower molecular weight units.

Eriksson-Quensel and Svedberg (20) carried out a comprehensive study of hemocyanin from 22 species. In all their studies they found dissociation phenomena, both reversible and irreversible, which were usually irreversible at extremely low pH values. In general, dissociation occurred at pH values away from the isoelectric point, with the highest molecular weight occurring around the isoelectric point. They varied the ionic strength little (0.20 - 0.30 total molarity). Therefore, it is reasonable to conclude that pH was largely responsible for the association-dissociation.

Further studies by Svedberg (70) showed that while dillution increased dissociation, addition of ions suppressed it. Increased dissociation was also noted on adding amino acids. Serum albumin dissociated on adding thyroxin. He also noted that solutions high in salt concentration caused dissociation of thyroglobulin.

β-Glucuronidase is believed to dissociate if the very pure enzyme is diluted (8). The dissociation can be reversed by a number of proteins, nucleic acids, and other chemicals.

Insulin dissociates to a molecular weight of 12,000 below pH 2.0 and ionic strength less than 0.1 (83), but aggregates as the pH approaches 5.3 to a molecular weight of approximately 36,000. There is conflicting evidence as to whether or not insulin dissociates into a unit of molecular weight of 6,000. Fredricq and Neurath (21) found that it did but Tietze and Neurath (76) later found a molecular weight of 12,000 by light-scattering studies. Oncley, Ellenborgen, Gitlin, and Gurd (60) indicate that the dissociation

of insulin at low pH and low ionic strength is apparently due to mutual repulsion of the units. Evidence for this is a reduced charge on the protein as the pH increases and dissociation occurs to the dimer and trimer. A great deal of work has been done on this protein and has been reviewed by Edsall (19), Waugh (83), and others.

Two peaks, supposedly due to a dimerization, were found in serum albumin on saturation of the solutions with benzene (10).

Reichmann and Colvin (64) found that bovine plasma albumin was composed of at least three and probably four polypeptide chains held together by disulphide links. The splitting of bovine plasma albumin was carried out by oxidation with performic acid. Molecules of this type are held intact more strongly than those with weak bonds. It is generally considered that dissociating proteins are held together by electrostatic forces, van der Waal's forces, and by hydrophobic groups. The stronger disulphide links serve to show that protein molecules are not always a continuous polypeptide chain even though they may be stable under a number of conditions.

A great deal of work has been done by Johnson and co-workers on the association-dissociation systems of peanut globulin (Arachis hypogaea). Their work clearly illustrates equilibria phenomena in proteins.

As Johns and Jones (33) had claimed to have isolated two globulins which they named arachin and conarachin, Johnson (34) analyzed these proteins to ascertain whether or not they were mixtures or single proteins. Johnson (34) extracted globulins from the ground nut with 10% sodium chloride solution. The solution was then fractionated in two ways. Fraction A: The sodium chloride solution was diluted and

acidified to pH 5 with 0.1 N hydrochloric acid. Fraction B: The sodium chloride solution was 40% saturated with ammonium sulphate. Both fractions were called arachin by Johns and Horn (46). The precipitate of fraction A, when dispersed in 0.05 M phosphate buffer at pH 8 and analyzed in the ultracentrifuge, yielded two sedimenting peaks whose areas could be varied by methods of extracting the proteins. The precipitate of fraction B, in 0.05 M phosphate buffer at pH 8, yielded one sedimenting peak which had a sedimentation coefficient similar to the faster moving peak from fraction A. The conarachin was obtained by dialysis and saturation with ammonium sulphate of the filtrate from which arachin had been precipitated.

Johnson (35) did further studies on the A and B fractions on these proteins. Fraction A contained one $S^{14.6}$ and one $S^{9.5}$ component while fraction B contained one $S^{14.6}$ component. (To avoid confusion, the notation of Johnson (35) is used when referring to his work. That is, $S^{14.6}$ etc., refers to a component with a sedimentation coefficient of 14.6 Svedberg units.) Further evidence that this protein was an association-dissociation system was obtained by reprecipitation of fraction A from 10% sodium chloride by 40% saturation with ammonium sulphate to get a precipitate similar to fraction B in that it had only the $S^{14.6}$ component.

When A type precipitate was dispersed in ammonium sulphate at pH 8, there was a progressive disappearance of the $S^{9.5}$ component which seemed to reappear as the $S^{14.6}$ component as the ammonium sulphate concentration was increased to 20% of saturation.

Dilution and acidification to pH 5 of a phosphate buffer protein solution containing only $S^{14.6}$ component caused dissociation

of the molecule when the precipitate was taken up in 0.05 M phosphate buffer at pH 8. It was pointed out later by Johnson and Shooter (42) that a decrease in salt or protein concentration or both without acidification to pH 5, or a decrease in protein concentration and a lowering of pH to 5, was insufficient to cause dissociation. It must follow then, that it is essentially the reduction in salt concentration and lowering of pH to 5 which favors dissociation.

No appreciable change in concentration of $S^{14.6}$ or $S^{9.6}$ was observed in 0.05 M phosphate buffer at pH 8 on standing 120 days, nor did any $S^{9.5}$ appear in the $S^{14.6}$ material on standing for 35 days. It was therefore assumed that, at these pH levels and salt concentrations, the material was not necessarily in thermodynamic equilibrium, or at least the rate of attainment was very slow. At higher salt concentrations attainment of equilibrium occurred more rapidly but at pH values near 5, attainment of equilibrium was quite rapid in low salt concentrations.

Thus, low salt concentration, low pH, and low protein concentration favor dissociation of the arachin molecule, while the opposite conditions favor association. Johnson and Joubert (37) found that various ions increase association of arachin.

Johnson, Shooter, and Rideal (43) obtained very poor separation of the associated and dissociated peanut arachin electrophoretically. At the higher pH levels, the solution contained some 80% of dissociated $S^{9.0}$ species, this quantity decreasing at the lower pH levels until, at pH 7, the parent $S^{14.6}$ species predominated. They obtained qualitative agreement between the sedimentation

and electrophoretic results. The fastest migrating component was attributed to the $S^{14.6}$ species while the slower migrating component was attributed to the $S^{9.0}$ species.

Studies on the arachin systems using sodium dodecyl sulphonate ($NaSO_4C_{12}H_{15}$) detergent have been reported by Johnson and Joubert (37). At low sulphate concentrations, association of the arachin half-molecule is promoted with an efficiency increasing markedly with detergent chain length. They concluded that since ionic strength and pH have such marked effects, the interaction is partially electrostatic, but the effect of chain length indicates a definite contribution from nonpolar and van der Waal's forces. Light scattering studies have also been reported for the ground-nut globulins (9).

Edestin seems to be unstable below the isoelectric zone of about pH 5. Bailey (7) in using Osborne's nomenclature reports the appearance of edestan, a protean of $S^{\circ}_{20} = 2.6$ as compared with edestin of $S^{\circ}_{20} = 13.6$ at low pH levels. The dissociation was retarded by sulphate and oxalate which suggests that repulsion of positive charges is involved in the dissociation process (83). Edestin seems to be quite stable in solutions up to a pH level of 10 (25).

Excelsin was stable from pH 5.5 to 10 but at pH 11.9 the protein was completely disaggregated to units of about 1/6 that of normal excelsin. This latter disaggregation was reversible (13).

Soybean protein has been studied by various fractionating procedures and has been shown to be an association-dissociation

system (58). Like the peanut protein conarachin, the dissociated state of soybean protein is favored by high ionic strengths.

The seed protein of narras (Acanthosicyos horrida) which is a cucurbitaceous plant of South Africa, dissociated on complexing with sodium dodecyl sulphonate (49). This globulin-like protein could be reduced in size from a molecular weight of 343,000 to 110,000 and 56,000 by reducing the ionic strength and by sodium dodecyl sulphonate treatment.

Lupinus luteus seed protein can be converted almost quantitatively from a component of $S^{\circ}_{20} = 11.6$ in phosphate buffer at pH 7.0 to a component of $S^{\circ}_{20} = 7.2$ by dialysis against a borate buffer at pH 8.8 of lower ionic strength (48). Component of $S^{\circ}_{20} = 7.2$ could be converted to a component of $S^{\circ}_{20} = 11.6$ by raising the ionic strength and lowering the pH.

Both Lupinus angustifolius and Lupinus luteus seed protein show dissociation favored by low ionic strength and a high pH of approximately 7.

It has been found that the number of components as determined by electrophoretic analysis may differ from the number found by sedimentation. Soybean protein was found to be electrophoretically homogeneous by Briggs and Mann (11), but Naismith (58) found three sedimenting components in the ultracentrifuge. Although the material used by Briggs and Mann was probably purer than that used by Naismith, even this purer material did not show homogeneity by solubility methods. Naismith's material contained contaminating supernatant liquid, the proteins of which may be in sufficient concentration to appear on a sedimentation diagram.

Fuerst, McCalla, and Colvin (23) working with squash seed globulin found four components with S°_{20} values of 3.0, 7.1, 9.4, and 12.1. The system was studied at several pH levels below and above the isoelectric point. (The notation S^3, S^7, S^9, and S^{12} will be used for components with a sedimentation coefficient of 3.0, 7.1, 9.4, and 12.1, respectively.)

Components S^3 and S^7 were present at an ionic strength of approximately 0.03 - 0.02 and pH 3.5 - 4.5. There was a progressive increase in concentration of S^7 both with increased age and increased pH. The increase in concentration of S^7 appeared to accompany a decrease in S^3. Consequently, results obtained using dispersions of various concentrations of protein were used to plot log $[S^7]$ against log $[S^3]$, in an attempt to see whether the equation (2 or 3)$[S^3] \rightleftharpoons [S^7]$ was valid for this system. The data did not fit the two theoretically possible straight lines. Thus, no simple rapid equilibrium appeared to exist between the two components. Above pH 4.5, S^3 disappeared and heavy aggregates appeared. At pH 4.8, the highest pH used below the isoelectric range, only S^7 and the heavy aggregates were present. With 2% globulin in solution and 0.03 ionic strength acetate at pH 4.1, S^7 and S^9 components were present, but within 24 hours only S^9 was present. Components S^7 and S^{12} were present in preparations on the alkaline side of the isoelectric point. The solvent used was 0.1 M glycine, with the pH adjusted with sodium hydroxide. The concentration of S^{12} decreased as the pH increased. At pH 10.9, only a trace was present. When pH 11.4 was reached, the globulin dissociated into S^3 and S^7.

Fuerst et al. believe the system is one of association-dissociation. The most striking evidence for this is the reappearance of rapidly sedimenting protein from isolated S^3. The S^3 component, isolated using a separation cell, associated to a high polymer (or high polymers) with a change of pH from 4.1 to 4.8.

Sedimentation of the globulin in 10% sodium chloride solution gave a value of $S^{\circ}_{20} = 8.2$. The authors concluded that this was probably the same as component S^7 found in other solvents.

Temperature, apparently, had a very slight, if any, effect on the stability of S^7.

The authors conclude that S^7 is probably a dimer or trimer of S^3. Components S^9 contained one more monomer than did S^7, while S^{12} appeared to be a dimer of S^7. It is suggested that the component S^7 appearing on storage was different from the component S^7 produced immediately on dispersion. Most of the association-dissociation was irreversible, with a few limited reversible reactions.

The literature indicates that although comparative amino acid studies of the cucurbit seed globulins are extensive, little is known of their electrophoretic and sedimentation properties. The conclusions of Smith and Green (65) that the seed globulins from squash, pumpkin, cucumber, and watermelon show amino acid differences indicate that they might reasonably be expected to show differences in electrophoretic and sedimentation properties. The conclusions of Fuerst et al. on the sedimentation properties of squash seed globulin suggest the possibility that interesting association-dissociation phenomena may occur in other cucurbit seed globulins.

METHODS AND MATERIALS

Extraction and Crystallization

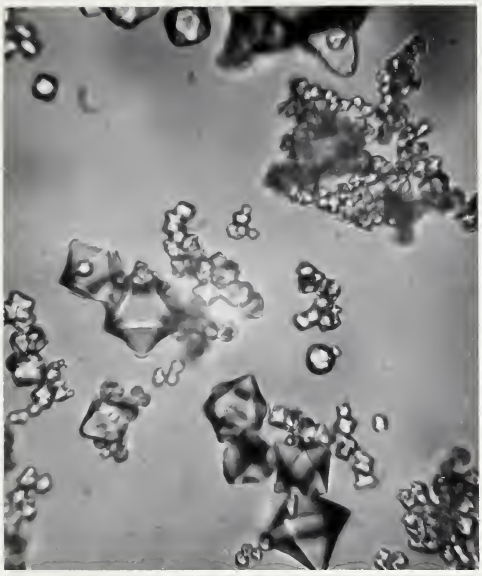
Seed was obtained from "The Desert Seed Company" of El Centro, California, through Capital Seeds Limited of Edmonton. The following species and varieties were used: Golden Hubbard Squash (Cucurbita maxima), Sugar Pie Pumpkin (Cucurbita pepo), Chicago Picking Cucumber (Cucumis sativus), and Klondyke Watermelon (Citrullus vulgaris).

Extraction and crystallization of the globulins from all varieties was done by the method of Fuerst (22) with two minor modifications. The fat extracted meal was not ball-milled and the globulin was extracted two hours instead of three hours with 10% sodium chloride. For recrystallization, the crystals were taken up in two-thirds the original volume of 10% sodium chloride extract.

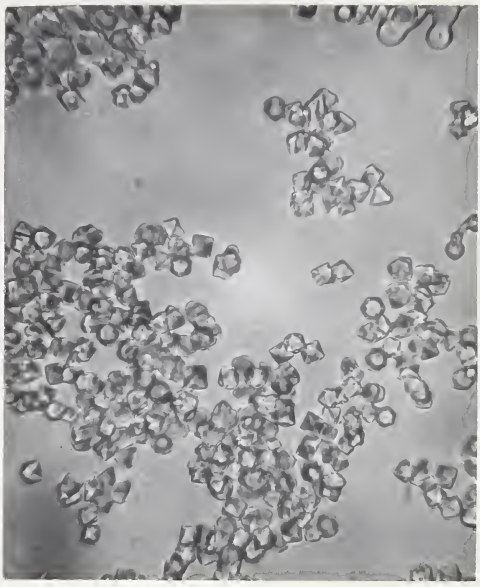
Fuerst et al. (23) heated the distilled water for diluting the crystals to 50°C, but Fuerst (22) heated it to 60°C. If the diluting water were heated to 50°C, the resulting 2% sodium chloride solution of protein frequently was not clear. This problem was eliminated by heating the diluting water to 60°C. The precipitated globulins were of the octahedral type and free from amorphous material as Fig. 1 shows.

It was found unnecessary to store the crystals under toluene as they were used before any visible fungal growth occurred.

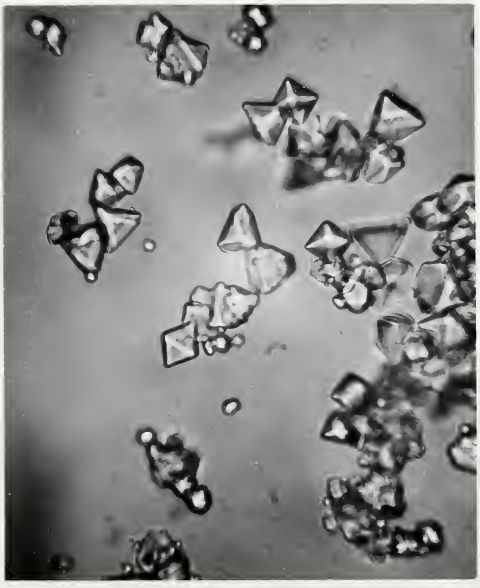
Danielson (18) criticizes the use of ether extraction in the preparation of seed globulins. According to him, it is unnecessary and it is not possible to say what effect boiling organic solvents have on the well defined protein.



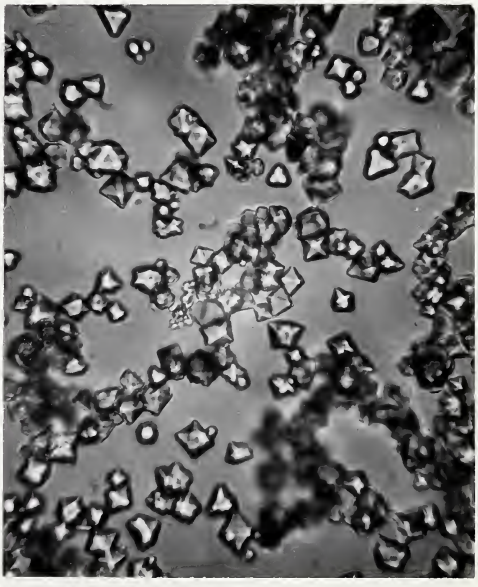
Squash globulin



Pumpkin globulin



Cucumber globulin



Watermelon globulin

Fig. 1

Crystalline globulin from cucurbit seeds

Approximate magnification 500 x.

Several reasons could be given for use of the fat extraction procedure in the preparation of seed proteins. Not only is the resulting flour more easily handled but more of the cells are disrupted.

When ether extraction is done with the soxhlet apparatus, protein never contacts boiling organic solvent during the ether extraction process, unless the temperature of the apparatus is hot enough to boil the ether in the meal-containing thimble. Ordinarily, only condensed solvent below the 35°C boiling point of ether would come in contact with the protein. The possible effects of organic solvents remain obscure, but ether-extraction has been commonly used in protein studies.

Preparation of Solutions for Electrophoresis and Sedimentation

In private communication, Dr. C. R. Fuerst states that he dispersed the crystals by removing an aliquot from the 2% sodium chloride mother liquor and after settling the crystals by low speed centrifugation, dispersed the pellet in the buffer solution. Solutions for electrophoretic analyses were dialyzed but solutions for sedimentation analyses were not.

A somewhat different method was adopted in the present study. The aliquot of crystals and mother liquor was drained from a pipette on to filter paper in a Buchner funnel. The mother liquor was removed by a slight reduction of the pressure under the funnel. The crystals were rinsed with distilled water, taking care to keep a layer of liquid on the crystals when applying the vacuum so that little or no air passed through them. Excess moisture was removed from the

moist cake of crystals by placing it between filter paper or paper towels and applying pressure with the hand. The moist cake contained four to five grams of water per gram of protein. Appropriate weights of the moist cake were dispersed in the desired solutions in two different ways, depending on the pH of the buffer used.

With the first method, the moist cake was dispersed directly in the buffer. With the second method the moist cake was first broken up in distilled water and then the buffer was added. The first method resulted in almost clear dispersions from pH 1.4 to pH 4.0 but resulted in a great deal of opalescence with dispersions from pH 4.0 to pH 4.8. The second method was therefore used from pH 4.0 to pH 4.8 as almost clear dispersions were obtained by it. Since solubility was poor at pH levels above the isoelectric point, it was immaterial which method of dispersion was used as opalescence occurred in either case. All solutions showing marked opalescence were largely cleared by low speed centrifuging. The nondispersed material was insoluble in a number of buffers and 10% sodium chloride but dissolved in approximately 50% acetic acid. Thus, this material probably largely consisted of denatured protein.

Buffers were made from reagent grade chemicals. In each case the molarity was adjusted with the salt and sufficient base or acid was added to adjust the pH. The pH of the buffer changed from one to two tenths of a unit with protein dispersion. In each case the pH reported is the pH of the final protein solution. Buffer pairs were the following:

1. pH 1 - 3.5, glycine with pH adjusted with hydrochloric acid.
2. pH 1 - 3.5, sodium formate with pH adjusted with hydrochloric acid.

3. pH 2.- 3, potassium chloride with pH adjusted with hydrochloric acid.
4. pH 2 - 3, sodium phosphate with pH adjusted with hydrochloric acid.
5. pH 3.5 - 4.8, sodium acetate with pH adjusted with acetic acid.
6. pH 7 - 12, glycine with pH adjusted with sodium hydroxide.
7. pH 5 - 10, sodium chloride with pH adjusted with sodium hydroxide.
8. pH 10 - 11, sodium phosphate with pH adjusted with sodium hydroxide.

To be consistent with the terminology used in this laboratory, the term "dispersion" rather than "solution" is used throughout this thesis.

Dispersions for electrophoretic analyses were dialyzed for three days under static dialysis or for at least 24 hours by mechanical dialysis. The dispersions were dialyzed against the actual buffer solutions to be used in the electrophoresis apparatus.

For mechanical dialysis, the dispersions were placed in dialyzing tubing which was tied at both ends. The dialysis tubes were then suspended, from the rotating shaft of a motor, in the buffer against which they were to be dialyzed. There was enough irregularity in the rotation so that the protein solution within the dialyzing tubing was continually swirled. The time required for dialysis can be reduced two to three times by mechanical movement of the membrane and solutions (75).

Dispersions used in the ultracentrifuge were centrifuged at low speed to remove opalescence, if present, but were not dialyzed.

Danielson (18) comments that the method of dispersing the crystals as used by Fuerst et al. is unsatisfactory for dissociating systems. The aggregate may be different in solubility from the

dissociated product and where dispersion is not complete there is likely to be differential dispersion of aggregated and dissociated fractions. Joubert (47) dispersed the proteins at weak ionic strength and then dialyzed them against buffer of the desired concentration and pH, taking care that no protein precipitated. It should be said that, as only trace amounts of the cucurbit globulin precipitated at pH levels below the isoelectric point by the method of dispersal used in this work, Danielson's criticisms are not serious. Even so, to study the cucurbit globulins below the isoelectric point under conditions such that no protein precipitation occurred would require salt concentrations lower than 0.01 M which would make sedimentation coefficients of the highly charged protein of doubtful value. A dispersion to be analyzed immediately cannot be prepared by dialysis as dialysis requires considerable time. The cucurbit globulins are very sensitive to salt concentration. Consequently, it would be uncertain what effect the prolonged pretreatment at low ionic strength would have on the protein. In order to have results at all comparable with regard to age, it is essential that dispersal methods not requiring prolonged treatment at low ionic strength must be used.

Hydrogen ion concentration was measured with a Beckman pH meter, Model G, to pH $\pm$ 0.05. For pH values over 10, the Beckman Type "E" glass electrode was used. Sodium ion corrections are not required with this electrode for 0.1 M solutions until a pH of 12 is reached.

Electrophoresis

The Klett Electrophoresis Apparatus was used. This apparatus is similar to one designed by Tiselius (77) and modified by Longsworth (54). The instrument uses a schlieren-scanning technique as described by Longsworth and MacInnes (56). Current to the apparatus was controlled by a "Heathkit Variable Voltage Regulated Power Supply". This unit supplies power from 0 - 500 volts and from 0 - 200 milliamperes with a current regulated from ± 1% to ± 3%, depending on the amount of current being used. If adjustments are made periodically, a current regulated to ± 0.5% can be obtained.

Conductivity measurements were made with a Conductivity Bridge Model RC-1 manufactured for Industrial Instruments, Inc. This instrument is accurate to ± 1.0% if properly used.

Electrophoretic analyses were carried out at 20°C because of the low solubility of the globulin at low temperatures. According to Johnson and Shooter (41), electrophoresis at 4°C and 20°C give identical results. Fuerst (22) also found identical electrophoretic results at 4°C and 20°C. At this higher temperature, the current must be reduced to avoid convections due to heating. This results in longer electrophoretic experiments and greater diffusion during an experiment.

Longsworth (54), Longsworth and McInnes (56), Alberty (2, 3), and Abramson *et al.* (1) give good descriptions of the theory and experimental application.

Calculations used were those described by Alberty (2).

$$u = \frac{xqk_p}{it}$$

u = electrophoretic mobility in cm. volt^{-1} sec^{-1}.

x = distance the descending boundary moves in cm.

q = cross sectional area of the electrophoresis cell in cm^2.

k_p = conductivity of the protein solution in mhos cm^{-1} at 20°C.

i = current in amperes.

t = time in seconds.

No attempt was made to divide the descending boundary into equal areas as suggested by Alberty (2). Where skewing occurred, the measurement was taken at the maximum ordinate or if two components appeared the more rapidly migrating peak was measured.

Sedimentation

The ultracentrifuge used throughout these studies was a Model E Spinco Ultracentrifuge, Serial No. 192. The instrument has an electrically driven 6.50 cm. radius rotor which developed, in the cell, forces up to 250,000 times that of gravity at 59,870 r.p.m.

The temperature is measured to $\pm$ 0.2°C before and after the run with a thermocouple inserted in the bottom of the rotor. As the rotor spins under reduced air pressure, little heating by friction occurs. The drive shaft made of one-tenth inch piano wire reduces conduction of heat from the motor to the rotor.

The optical system is of the Philpot (63) Svensson (72) type. The refractive index gradient caused by the sedimenting protein deviates a beam of light. The deviated beam is refracted by a cylindrical lens proportionally to the degree of deviation in the cell. The photograph of the shadow cast by the schlieren bar is taken automatically. A filter allows only green-yellow light to be effective.

Sedimentation coefficient measurements:

According to Svedberg and Pedersen (71), sedimentation is represented by $s = \frac{dx/dT}{w^2x}$ which on integration gives $s = \frac{\ln(x_2/x_1)}{w^2(T_2 - T_2)}$.

A close approximation for values of $x_2/x_1 < 1.4$ is

$s = \frac{2(x_2 - x_1)}{(x_2 + x_1)w^2(T_2 - T_1)}$. Sedimentation coefficients corrected to water at 20°C become

$$S^{\circ}_{20} = \frac{2(x_2 - x_1)}{(x_2 + x_1)w^2(T_2 - T_1)} \cdot \frac{\eta t^{\circ}}{\eta 20^{\circ}} \cdot \frac{\eta t^{solv.}}{\eta t^{\circ}} \cdot \frac{(1 - \bar{v}_{20^{\circ}} \rho_{20^{\circ}})}{(1 - \bar{v}t\, \rho t^{soln.})} .$$

S°_{20} = sedimentation coefficient corrected to water at 20°C.

x_2 = distance in cm. of peak from center of rotation at time T_2.

x_1 = distance in cm. of peak from center of rotation at time T_1.

w = velocity of the rotor in radians per second.

T_2 and T_1 = time in seconds at time of measurement.

ηt° = viscosity of water at t°C.

$\eta 20^{\circ}$ = viscosity of water at 20°C.

$\eta t^{solv.}$ = viscosity of solvent at t°C.

$\bar{v}_{20}$ = partial specific volume of the protein in buffer at 20°C.

$\bar{v}t$ = partial specific volume of the protein in buffer at t°C.

$\rho 20^{\circ}$ = density of solvent at 20°C.

ρt° = density of solvent at t°C.

The latter formula was used with these limitations: $\frac{\eta_{t^\circ}}{\eta_{20^\circ}}$ was evaluated from tables in Svedberg et al. (71). $\frac{\eta_{t^{solv.}}}{\eta_{t^\circ}}$ was ordinarily < 1.02 and was omitted unless the value was found to be > 1.02. $\frac{(1 - \bar{v}_{20}\rho_{20^\circ})}{(1 - \bar{v}_t\rho_t)}$ was assumed to be 1.00. No attempt was made to correct sedimentation coefficients to zero protein concentration.

Sedimentation coefficients were determined for each of the intervals photographed and then averaged. The viscosity correction was applied to the average except where the temperature rise during a run was greater than 1°C. In this case, the viscosity correction was applied for the initial temperature, average temperature, and final temperature of the rotor during the run. The corrected sedimentation coefficients were then averaged. The rotor undergoes an adiabatic cooling of 1°C (84) on acceleration to 59,780 r.p.m. This correction was included.

The various factors and constants used in calculation of sedimentation coefficients were checked and found to be in close agreement with those supplied by the Spinco Company and with those found by Taylor (73) for a Spinco Model E Ultracentrifuge. The accuracy of sedimentation coefficients has been discussed by Cecil and Ogston (15), Taylor (73), Oncley (59), and Kegeles and Gutter (51).

The theory of sedimentation and physical measurements of proteins in dilute solutions are discussed by Svedberg and Pedersen (71), Alexander and Johnson (4), and Oncley (59).

RESULTS

Solubility of the Cucurbit Globulins

No attempt was made to establish the quantitative solubility characteristics of the cucurbit globulins, but observations were made throughout the work on the qualitative solubility from the degree of opalescence.

Dispersions from all four globulin species were satisfactory at pH values below 4.3 and salt concentrations below 0.03 M. Cucumber and watermelon globulin did not disperse as readily as squash and pumpkin globulin. Dispersion increased progressively in opalescence as the pH of the dispersing agent increased to pH 4.8.

Watermelon globulin was the color of a dilute colloidal ferric hydroxide solution. The coloring became more intense after dispersion and with age. Although the coloring was present at all pH levels, it was always a very light shade. The degree of coloring of the crystals decreased after each crystallization until four crystallizations were completed. As seven crystallizations caused no appreciable decrease in the coloring as compared with material recrystallized four times, the colored material must have had a solubility very nearly the same as the crystals or it must have been adsorbed on the protein. This discussion indicates that the colored material is probably not an integral part of the crystalline protein. As the colored material probably came from the seed coats, the most reasonable explanation for its presence in recrystallized globulin is adsorption of the material on the globulin. No further

attempt was made to characterize or reduce the quantity of the colored material, nor was globulin prepared after removal of the seed coats.

Joubert (47), in his recent studies of dissociation in Lupin seed protein, obtained interesting results after colored material was removed from this protein. Prior to removal of the yellow colored material, diagrams from electrophoretic analyses showed one more component than diagrams from sedimentation analyses. This was taken to mean that the yellow contaminant modified the electrophoretic porperties of the proteins, but was not heavy enough to modify the sedimentation coefficient. As no results directly comparable with those of Joubert were found in the present studies, his findings probably are not pertinent to this work.

More complete dispersal of the globulins was achieved when they were in the form of separated crystals than when the crystals were packed together into hard cakes. If the crystals were caked, the protein became "gummy" before it dispersed. This phenomenon was especially evident at pH values above 4.0. The tendency for gumminess was greater in cucumber and watermelon globulin than in squash and pumpkin globulin. The nondispersed material occurring in some preparations was insoluble in a number of solvents that normally dissolve these globulins and therefore probably represented denatured protein.

While above pH 6, dispersions of globulin from the four species were clear, all became opalescent in 10% sodium chloride solution as the pH was decreased below this point. Between pH 4.9

and 5.0, the opalescence changed to cloudiness. At pH 4.8 precipitation occurred. Between pH 4.8 and 5.0 there was a change in the solubility characteristics of the globulins. Below these pH levels the globulins were slightly soluble in 0.1 M salt solutions and almost completely soluble in 0.04 M salt solutions, while above these pH levels the globulins were only soluble in 1.7 M salt solutions and higher. Dispersions of protein in 0.1 M salt solution showed marked opalescence even at pH 8.0 - 9.0 and did not become clear until a pH of 11.0 was reached.

Electrophoretic Analyses

In the following electrophoretic diagrams, the descending boundary is indicated by "→ Desc." and the ascending boundary by "Asc. ← ".

According to Johnson and Shooter (41) convection effects due to heating may appear as sharp peaks migrating with the boundary. To avoid convection effects at 20°C, it is generally agreed the maximum field strength should be one-third of the value used at 0°C. Thus, if energy dissipation equivalent to 0.15 volts/cm^3 (2) can occur without convection effects, the convection-free field strength can be calculated using:

$$E = \frac{1}{3}\sqrt{\frac{H}{K}}$$

E = field strength in volts/cm.
H = volts/cm^3.
K = mhos/cm.

In most cases the field strength used was at or near this maximum. It is possible that some convection phenomena did occur but, if the electrophoresis run had been prolonged by reducing the field strength, diffusion might in some cases have masked the resolution that was obtained.

The sharp peak in the descending boundary of Fig. 7 may be due to convection effects. Other diagrams showing sharp peaks which may be due to convection effects are found in Fig. 20, 22, and 31. In each case, however, photographs taken at different times were used to make the identification of the number of components in the system accurate.

Squash Seed Globulin

A check of some of the electrophoretic experiments made by Fuerst (22) on squash seed globulin produced similar characteristic diagrams. While only one peak occurred in 0.05 M sodium acetate at pH 3.9, two distinct components appeared in the same buffer at pH 4.8 (Fig. 2).

In an attempt to find the pH at which the second component appeared, preparations between pH 3.9 and 4.8 were studied. One component was found at each of pH 4.3 (diagram omitted) and pH 4.6 (Fig. 3). The second electrophoretic component therefore appeared within the very narrow range of 0.2 pH units.

In the course of these investigations, this protein appeared to be sensitive to small variations in salt concentration. Experiments in 0.01 M sodium acetate at pH 4.8 (Fig. 4) and those in 0.01 M sodium acetate at pH 4.3 (Fig 5) gave results similar to those in 0.05 M sodium acetate. This is in distinct contrast to results for pumpkin, cucumber, and watermelon globulin to be presented later.

Figures 6 and 7 present other interesting results found at low pH values. In both 0.04 M glycine at pH 2.3 and 0.05 potassium chloride at pH 2.2, the diagrams showed two components. The sharp peak

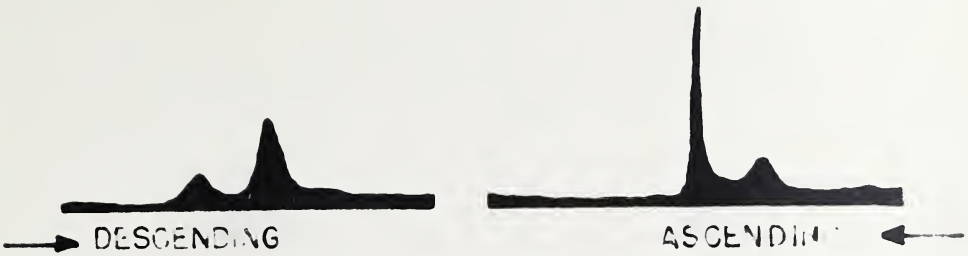


Fig. 2

Squash globulin in 0.05 M sodium acetate.
Time 151 minutes. Protein conc. 0.4%. Magnification x 1.
Descending: pH 4.8. Field strength 1.7 volts/cm.
Ascending: pH 4.8. Field strength 1.7 volts/cm.

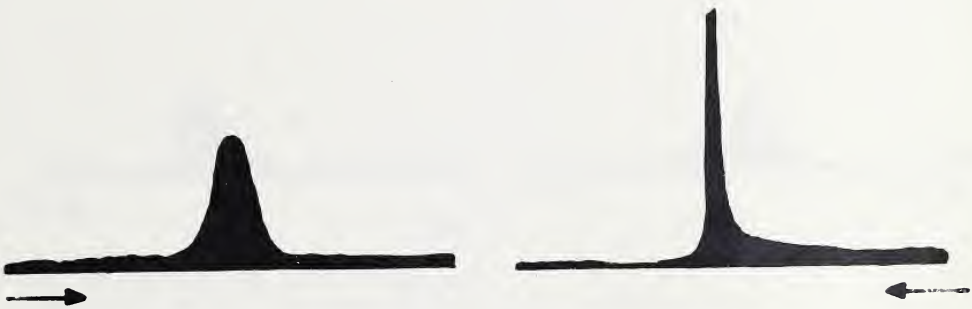


Fig. 3

Squash globulin in 0.05 M sodium acetate.
Time 244 minutes. Protein conc. 0.61%. Magnification x 1.
Descending: pH 4.6. Field strength 2.3 volts/cm.
Ascending: pH 4.6. Field strength 2.1 volts/cm.

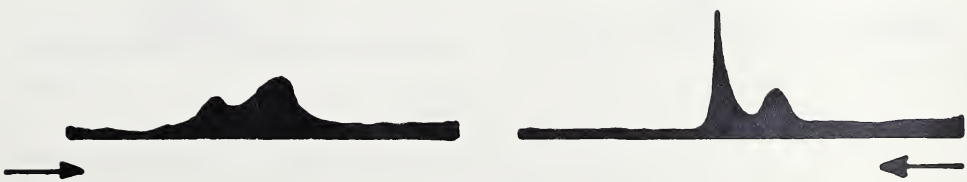


Fig. 4

Squash globulin in 0.01 M sodium acetate.
Time 80 minutes. Protein conc. 0.38%. Magnification x 1.
Descending: pH 4.8. Field strength 3.4 volts/cm.
Ascending: pH 4.8. Field strength 3.6 volts/cm.

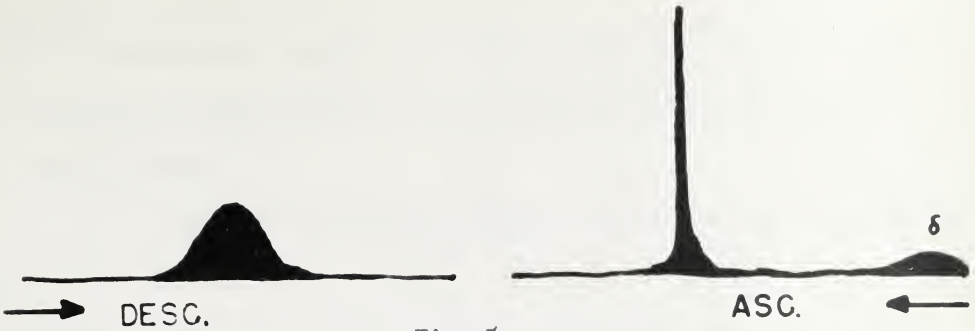


Fig. 5

Squash globulin in 0.01 M sodium acetate.
Time 95 minutes. Protein conc. 0.55%. Magnification x 1.
Descending: pH 4.3. Field strength 3.1 volts/cm.
Ascending; pH 4.3. Field strength 3.2 volts/cm.

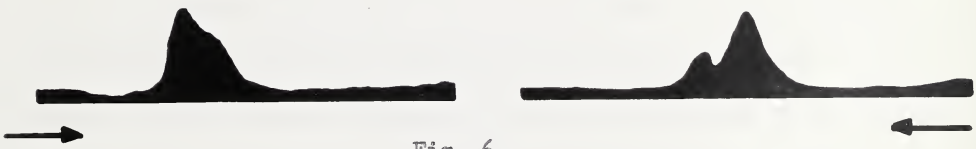


Fig. 6

Squash globulin in 0.04 M glycine.
Time 285 minutes. Protein conc. 0.42%. Magnification x 1.
Descending: pH 2.3. Field strength 1.1 volts/cm.
Ascending: pH 2.3. Field strength 1.2 volts/cm.

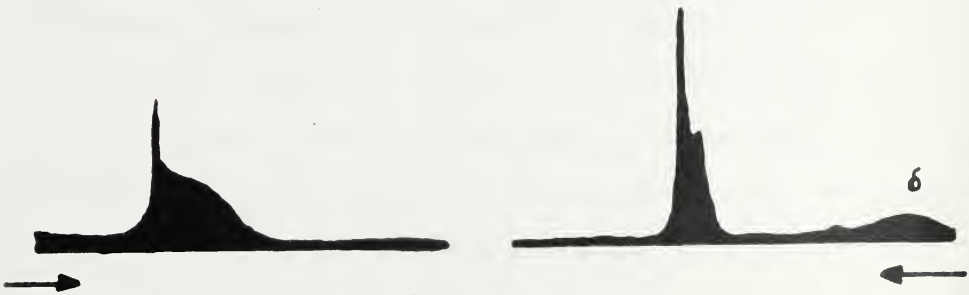


Fig. 7

Squash globulin in 0.05 M potassium chloride.
Time 209 minutes. Protein conc. 0.46%. Magnification x 1.
Descending: pH 2.3. Field strength 1.4 volts/cm.
Ascending: pH 2.3. Field strength 1.4 volts/cm.

in the descending boundary may be due to heating effects. Complete separation of these components was not obtained even after 285 and 320 minutes.

Above the isoelectric point, the globulin was investigated in 0.05 M glycine sodium hydroxide buffer at pH 9.1 and pH 10.1. Again resolution was poor as only the descending boundary indicated two components. This phenomenon also occurred in 0.1 M glycine sodium hydroxide at pH 10.1 (Fig. 8).

Of interest is the fact that squash globulin yielded two components at pH 10.0 - 10.1. But unpublished work by Fuerst showed only one component at pH 10.2 in 0.1 M glycine buffer. The same unpublished work shows two components in 0.1 M glycine buffer at pH 9.9. There therefore appears to be a critical point from pH 10.1 to 10.2 for this protein. A similar phenomenon occurred from pH 4.8 to pH 4.6.

With 0.05 M glycine buffer at pH 10.6, both the ascending and descending boundaries were symmetrical (Fig. 9). The very irregular base line makes it difficult to say whether or not this protein is electrophoretically homogeneous, but if components of different mobilities do exist, they are in trace amounts. The sharp peaks found in Fig. 9 usually occur when the field strength is too high.

Pumpkin Seed Globulin

Dispersions of pumpkin seed globulin at various pH values were studied in various buffers. Two distinct components appeared

Fig. 8

Squash globulin in 0.10 M glycine.
Time 286 minutes. Protein conc. 0.41%. Magnification x 1.
Descending: pH 10.1. Field strength 1.9 volts/cm.
Ascending: pH 10.1. Field strength 1.9 volts/cm.

Fig. 9

Squash globulin in 0.05 M glycine.
Time 212 minutes. Protein conc. 0.81%. Magnification x 1.
Descending: pH 10.5. Field strength 2.7 volts/cm.
Ascending: pH 10.6. Field strength 2.7 volts/cm.

at pH 4.8 and 0.10 M (Fig. 10) and 0.05 M sodium acetate buffer (Fig. 11). One main component appeared at pH 4.6 (Fig. 12) with a possible trace amount of a second component. The asymmetry of the diagrams in Fig. 13 suggest that there were two components in 0.01 M sodium acetate at pH 4.3. There is no such evidence in the preparation in 0.05 M sodium acetate at pH 4.2 (Fig. 14).

In 0.04 glycine buffer at pH 2.3, the globulin did not completely separate into two peaks but there is an indication of two electrophoretically different components (Fig. 15). Another experiment was carried out under similar conditions but the results were no more definite than those in Fig. 15. In a solution of 0.05 N potassium chloride at pH 2.2 (Fig. 16), the two components were more clearly indicated as the peaks appeared strongly skewed in the ascending and descending boundaries. Only one electrophoretic peak developed in 0.04 M glycine at pH 1.2.

Although the ascending boundary was only slightly skewed in 0.05 M glycine buffer at pH 10.0, the descending boundary separated into two distinct peaks (Fig. 17). Longworth (55) gives an interpretation for sharper descending than ascending boundaries. While normally the ascending boundary gives sharper peaks, the reverse was evident in these diagrams.

Cucumber Seed Globulin

Various pH values and various buffers were used to characterize cucumber seed globulin. Figure 18 shows that the globulin in 0.01 N sodium acetate at pH 4.8 gave one predominant component. Another preparation of crystals in 0.05 N sodium acetate

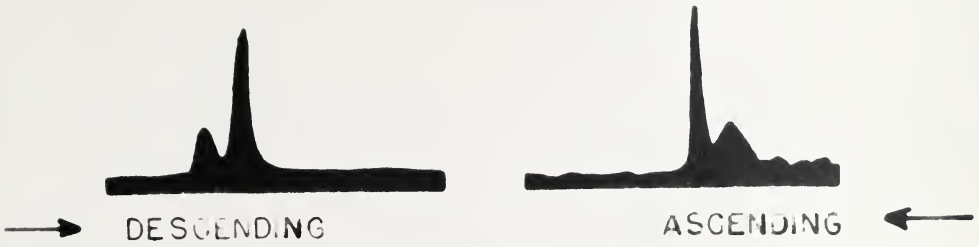


Fig. 10

Pumpkin globulin in 0.10 M sodium acetate.
Time 230 minutes. Protein conc. 0.38%. Magnification x 1.
Descending: pH 4.74. Field strength 1.2 volts/cm.
Ascending: pH 4.75. Field strength 1.2 volts/cm.

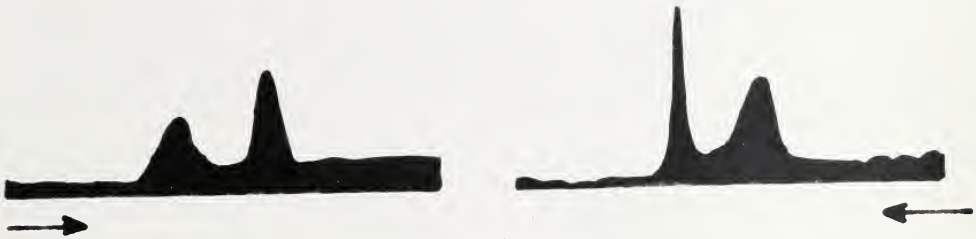


Fig. 11

Pumpkin globulin in 0.05 M sodium acetate.
Time 230 minutes. Protein conc. 0.62%. Magnification x 1.
Descending: pH 4.80. Field strength 2.9 volts/cm.
Ascending: pH 4.78. Field strength 3.4 volts/cm.

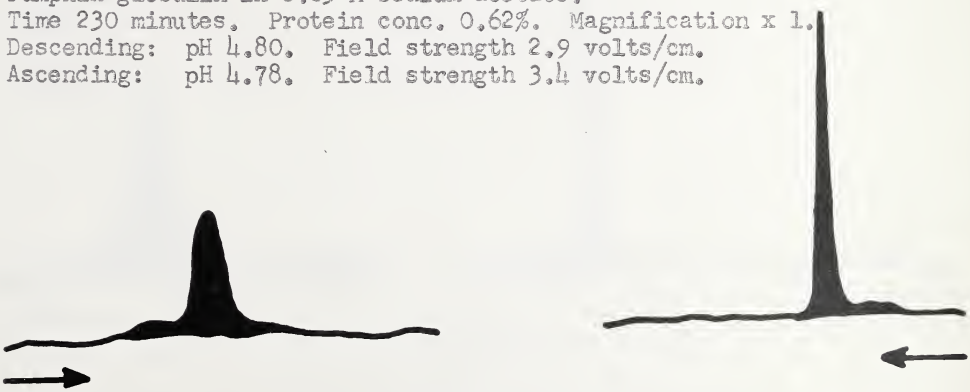


Fig. 12

Pumpkin globulin in 0.05 M sodium acetate.
Time 205 minutes. Protein conc. 0.45%. Magnification x 1.
Descending: pH 4.6. Field strength 2.3 volts/cm.
Ascending: pH 4.6. Field strength 2.3 volts/cm.

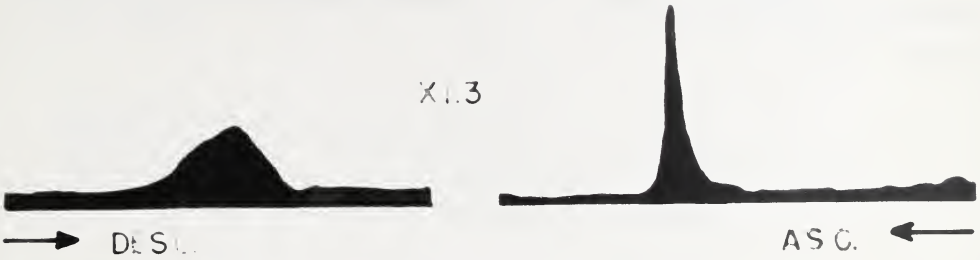


Fig. 13

Pumpkin globulin in 0.01 M sodium acetate.
Time 99 minutes. Protein conc. 0.30%. Magnification x 1.3.
Descending: pH 4.3. Field strength 2.5 volts/cm.
Ascending: pH 4.3. Field strength 2.9 volts/cm.

Fig. 14

Pumpkin globulin in 0.05 M sodium acetate.
Time 240 minutes. Protein conc. 0.47%. Magnification x 1.3.
Descending: pH 4.2. Field strength 2.3 volts/cm.
Ascending: pH 4.2. Field strength 2.2 volts/cm.

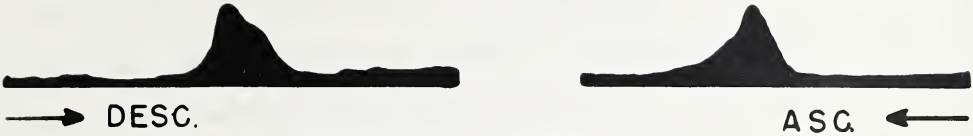


Fig. 15

Pumpkin globulin in 0.04 M glycine.
Time 179 minutes. Protein conc. 0.37%. Magnification x 1.
Descending: pH 2.3. Field strength 1.9 volts/cm.
Ascending: pH 2.3. Field strength 1.8 volts/cm.

Fig. 16

Pumpkin globulin in 0.05 M potassium chloride.
Time 270 minutes. Protein conc. 0.50%. Magnification x 1.
Descending: pH 2.2. Field strength 1.5 volts/cm.
Ascending: pH 2.2. Field strength 1.4 volts/cm.

Fig. 17

Pumpkin globulin in 0.05 M glycine.
Time 273 minutes. Protein conc. 0.20%. Magnification x 1.
Descending: pH 10.0. Field strength 2.9 volts/cm.
Ascending: pH 10.0. Field strength 2.9 volts/cm.

at pH 4.8 gave only one distinguishable component. A small amount of a second component appeared in the ascending boundary with 0.01 M sodium acetate at pH 4.3 (Fig. 19) but in 0.05 M sodium acetate at pH 4.2 (Fig. 20) only one slightly skewed peak appeared. The small sharp peak probably has no significance. When the pH was lowered to 2.3, the diagram of a dispersion, for the ascending boundary, showed two distinct but poorly separated components while the descending boundary was definitely skewed (Fig. 21). An experiment in 0.05 M potassium chloride at pH 2.2 (Fig. 22) showed a diagram with marked skewing in the descending boundary which may represent two components. Here, too, the small sharp peak in the descending boundary of Fig. 22 is probably due to convection effects.

Above the isoelectric point, two components separated at all the pH values investigated. Separation occurred in 0.05 M glycine buffer at pH 10.0 in both ascending and descending boundaries (Fig. 23) but in 0.05 M glycine buffer at pH 10.5 (Fig. 24) and pH 9.2 separation occurred only in the descending boundary.

Watermelon Seed Globulin

Watermelon globulin in various buffers at various pH values showed considerable variability. Only single boundaries were found at pH 4.7 in 0.01 M (Fig. 25) and at pH 4.8 in 0.05 M sodium acetate buffers. A second component appeared in 0.01 M sodium acetate at pH 4.3 (Fig. 26) but probably did not occur in 0.05 M sodium acetate at pH 4.2 (Fig. 27). In both 0.03 M and 0.01 M sodium acetate at pH 4.0, two components seem to be present, the

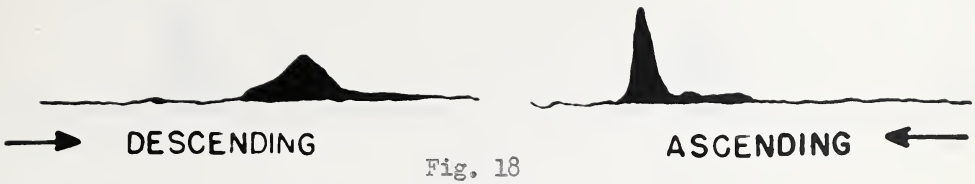


Fig. 18

Cucumber globulin in 0.01 M sodium acetate.
Time 100 minutes. Protein conc. 0.39%. Magnification x 1.
Descending: pH 4.8. Field strength 1.3 volts/cm.
Ascending: pH 4.8. Field strength 1.4 volts/cm.

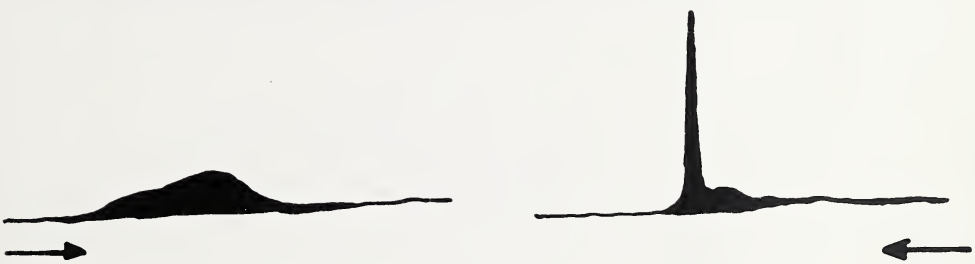


Fig. 19

Cucumber globulin in 0.01 M sodium acetate.
Time 165 minutes. Protein conc. 0.39%. Magnification x 1.
Descending: pH 4.3. Field strength 3.0 volts/cm.
Ascending: pH 4.2. Field strength 3.1 volts/cm.

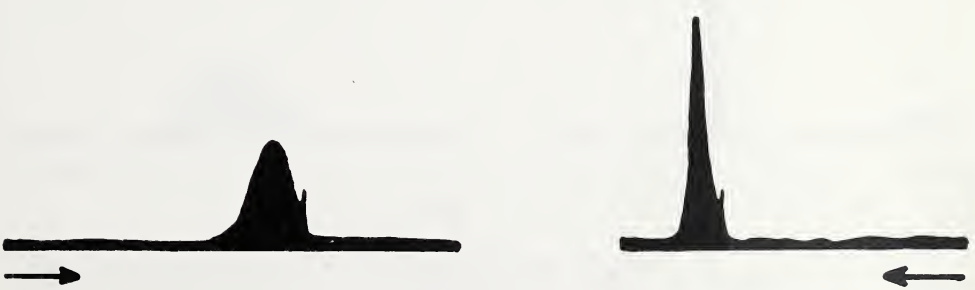


Fig. 20

Cucumber globulin in 0.05 M sodium acetate.
Time 252 minutes. Protein conc. 0.37%. Magnification x 1.
Descending: pH 4.1. Field strength 2.2 volts/cm.
Ascending: pH 4.2. Field strength 2.2 volts/cm.

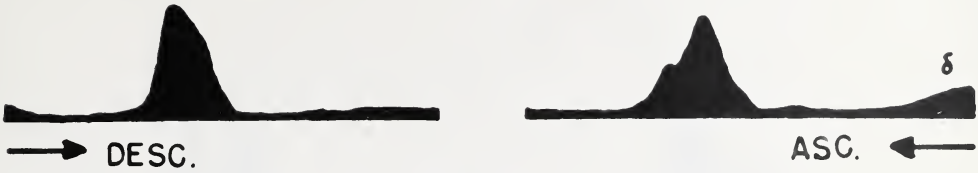


Fig. 21

Cucumber globulin in 0.04 M glycine.
Time 185 minutes. Protein conc. 0.41%. Magnification x 1.
Descending: pH 2.3. Field strength 1.7 volts/cm.
Ascending: pH 2.3. Field strength 1.7 volts/cm.

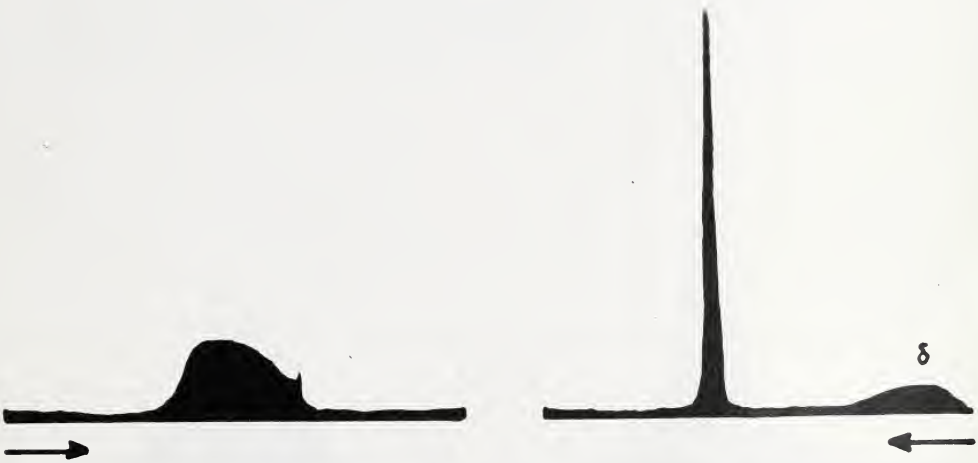


Fig. 22

Cucumber globulin in 0.05 M potassium chloride.
Desc. time 243 minutes. Asc. time 188 minutes. Protein conc. 0.63%.
Magnification x 1.
Descending: pH 2.2. Field strength 1.4 volts/cm.
Ascending: pH 2.2. Field strength 1.4 volts/cm.

Fig. 23

Cucumber globulin in 0.05 M glycine.
Time 181 minutes. Protein conc. 0.57%. Magnification x 2.
Descending: pH 10.0. Field strength 2.9 volts/cm.
Ascending: pH 10.0. Field strength 2.8 volts/cm.

Fig. 24

Cucumber globulin in 0.05 M glycine.
Time 267 minutes. Protein conc. 0.59%. Magnification x 2.
Descending: pH 10.5. Field strength 2.6 volts/cm.
Ascending: pH 10.5. Field strength 2.6 volts/cm.

Fig. 25

Watermelon globulin in 0.01 M sodium acetate.
Time 107 minutes. Protein conc. 0.58%. Magnification x 1.
Descending: pH 4.74. Field strength 3.5 volts/cm.
Ascending: pH 4.68. Field strength 3.4 volts/cm.

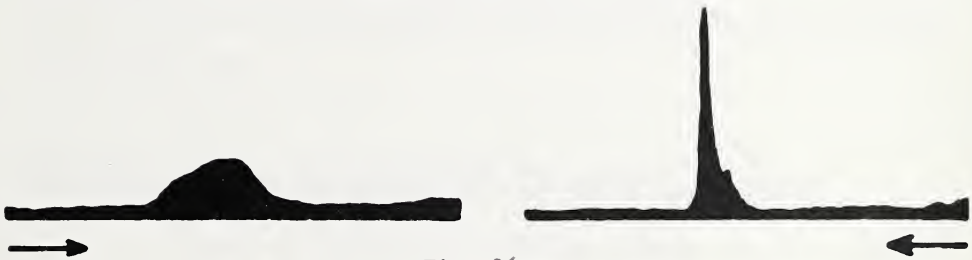


Fig. 26

Watermelon globulin in 0.01 M sodium acetate.
Time 120 minutes. Protein conc. 0.42%. Magnification x 1.
Descending: pH 4.3. Field strength 2.8 volts/cm.
Ascending: pH 4.3. Field strength 2.9 volts/cm.

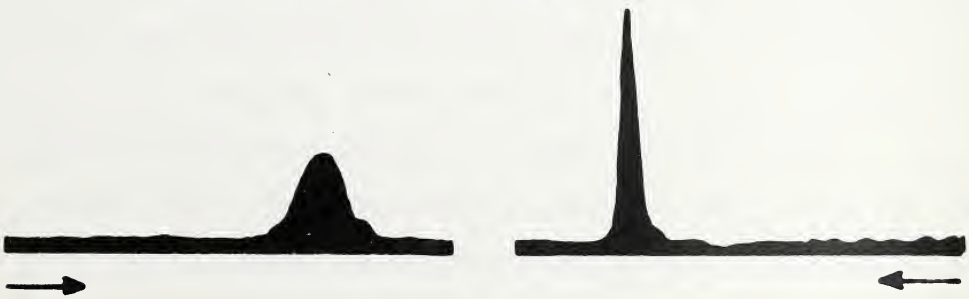


Fig. 27

Watermelon globulin in 0.05 M sodium acetate.
Time 278 minutes. Protein conc. 0.39%. Magnification x 1.
Descending: pH 4.2. Field strength 2.1 volts/cm.
Ascending: pH 4.2. Field strength 2.2 volts/cm.

0.01 M sodium acetate giving better resolution than the 0.03 M sodium acetate (Fig. 29 and 28).. Only one slightly skewed peak was present in 0.05 M sodium acetate at pH 3.9.

Both 0.04 M glycine and 0.05 M potassium chloride buffers at pH 2.3 and pH 2.2 gave two poorly resolved components (Fig. 30 and 31). The two components were even more poorly resolved in 0.04 M glycine buffer at pH 1.0. The sharp peak in the descending boundary of Fig. 31 is thought to be due to convection effects.

The descending boundary in 0.05 M glycine at pH 9.7 showed two definite peaks and the ascending boundary was definitely skewed (Fig. 32).

The Effect of pH on the Mobility of the Cucurbit Globulins

Figure 33 presents the mobility results for the globulins from each of the species plotted against pH. This is perhaps one of the more interesting phenomena of these studies. Although no great accuracy is claimed for the mobility results, they do serve to show that these proteins are quite similar with respect to the charge on the molecule. Mobilities are not only similar but they appear to increase and decrease in a similar fashion. It is recognized that a change in mobility could occur from a change in buffer alone. However, it is doubtful that this is the principle factor in determining these results, since watermelon globulin showed a decrease in mobility when the pH was lowered below pH 4.0 with sodium acetate buffer. Evidence that it was a pH effect is given by the lower mobilities of some preparations in both potassium

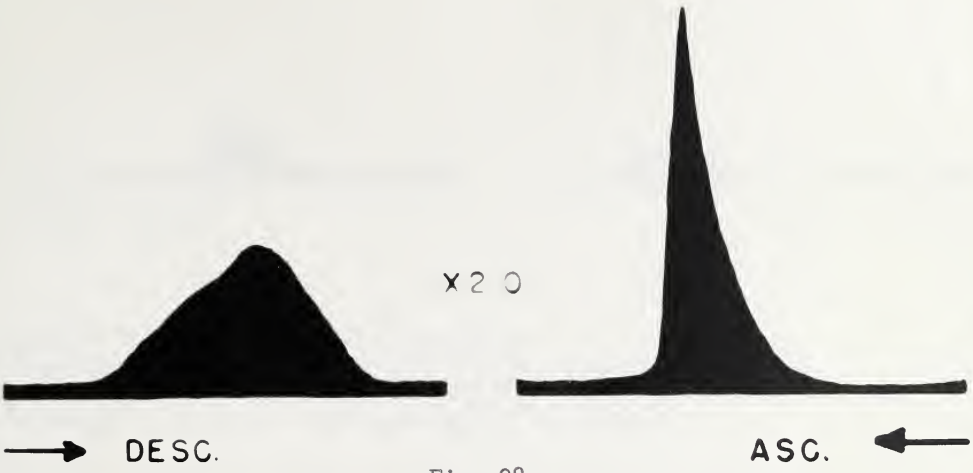


Fig. 28

Watermelon globulin in 0.03 M sodium acetate.
Time 218 minutes. Protein conc. 0.47%. Magnification x 2.
Descending: pH 4.0. Field strength 2.5 volts/cm.
Ascending: pH 4.0. Field strength 2.3 volts/cm.

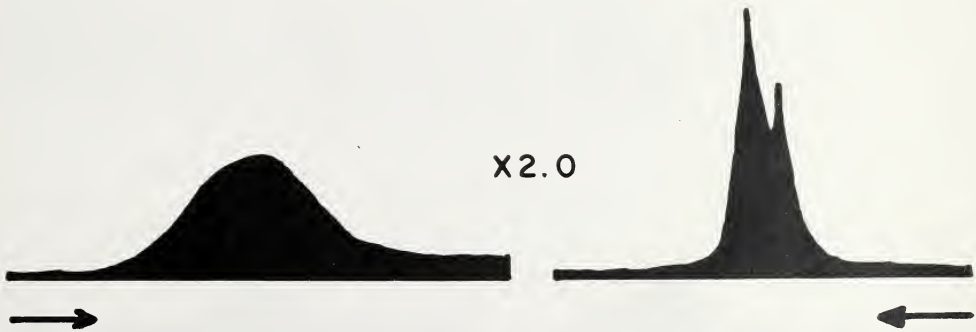


Fig. 29

Watermelon globulin in 0.01 sodium acetate.
Desc. time 80 minutes, Asc. time 99 minutes. Protein conc. 0.32%.
Magnification x 2.
Descending: pH 4.0. Field strength 2.9 volts/cm.
Ascending: pH 3.9. Field strength 3.2 volts/cm.

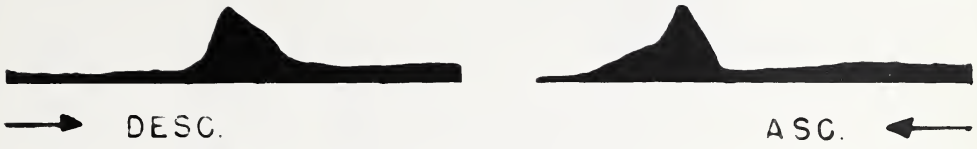


Fig. 30

Watermelon globulin in 0.04 M glycine.
Time 257 minutes. Protein conc. 0.42%. Magnification x 1.
Descending: pH 2.3. Field strength 1.8 volts/cm.
Ascending: pH 2.3. Field strength 1.8 volts/cm.

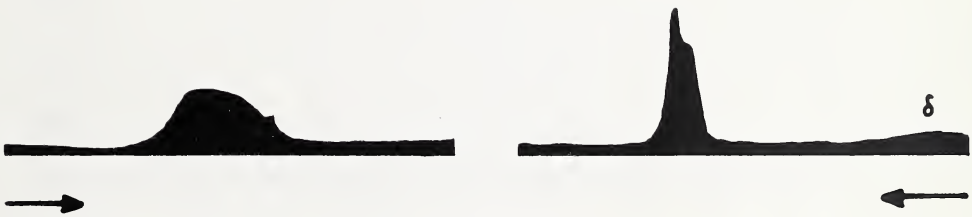


Fig. 31

Watermelon globulin in 0.05 potassium chloride.
Time 259 minutes. Protein conc. 0.40%. Magnification x 1.
Descending: pH 2.2. Field strength 1.4 volts/cm.
Ascending: pH 2.2. Field strength 1.4 volts/cm.

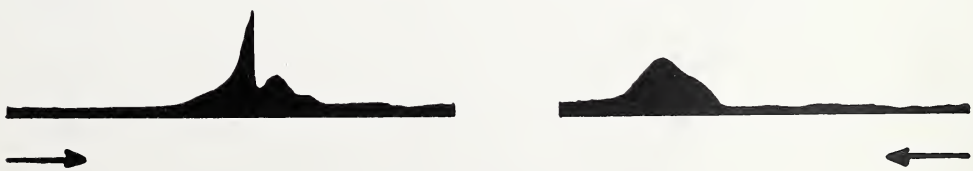


Fig. 32

Watermelon globulin in 0.05 M glycine.
Time 197 minutes. Protein conc. 0.27%. Magnification x 1.
Descending: pH 9.7. Field strength 2.9 volts/cm.
Ascending: pH --. Field strength 2.8 volts/cm.

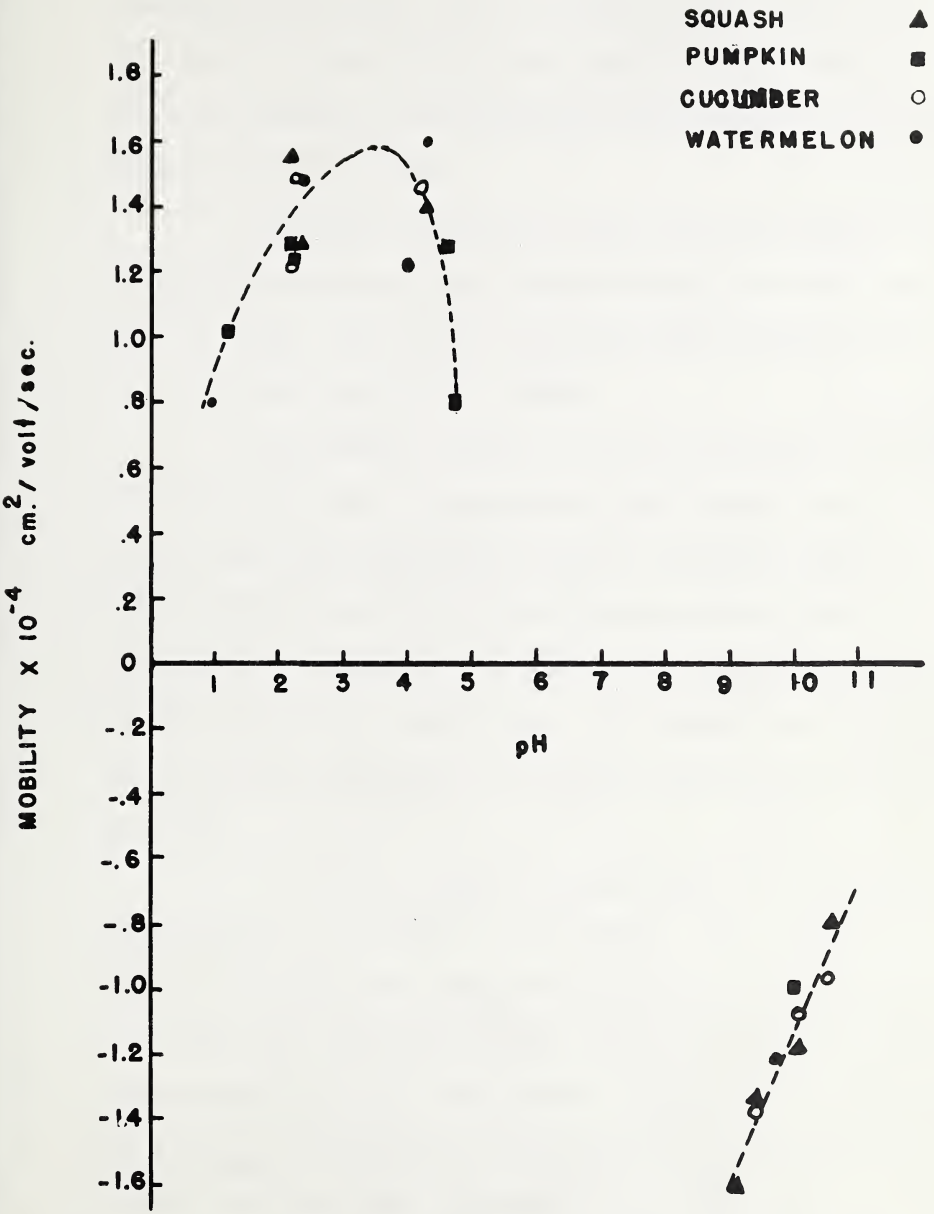


Fig. 33

The effect of pH on the mobility of the cucurbit globulins.

chloride and glycine buffer and in the still lower mobilities occurring at pH of 1.0 - 1.2 in glycine. Since only glycine and sodium hydroxide buffers were used above the isoelectric point, the decrease in mobilities was attributable to pH changes and not to buffer effects.

The high mobilities of these globulins indicate a high charge on the molecule at pH levels above and below the isoelectric point or range. This is to be expected with a protein with a high basic and acidic amino acid content.

The arginine content of the four species is from 16% to 18% (65). The values of glutamic acid and aspartic acid content for cucumber and squash globulin are high as reported by Thompson and Steward (74) who used the same varieties as those used by Smith and Green (65). The glutamic acid content of cucumber and squash globulin was 21.4% and 24.2%, respectively. The aspartic acid content was somewhat lower, being 9.3% for cucumber globulin and 6.8% for squash globulin.

Ordinarily, change in pH away from the isoelectric point should increase the charge on the protein. This appeared to occur at a pH of 3 - 4 below the isoelectric point and presumably at a pH of 6 - 9 above the isoelectric point, although insolubility precluded measurements below pH 9. The mobility decreased as the pH decreased below 3 - 4 and above pH 6 - 9 and thus the effective charge on the molecule must decrease. Any change in size or chape would not appreciably effect the mobility of the protein molecule as Alexander and Johnson (4) point out. The normally charged groups evidently

were rendered ineffective in some way. This could have been brought about by some form of inter or intra molecular rearrangement. As the sedimentation results show (next section), there is reason to believe that extensive spatial rearrangement of the molecule occurs below pH 4. Whether this is true or not above the isoelectric point remains to be tested.

Discussion of Electrophoretic Results

A summary of the electrophoretic results can be found in Table I. Differences among the globulins from the four species can be observed.

Evidence that the two components arise as a result of pH effects comes from the fact that each globulin gives similar results in glycine and potassium chloride buffers at pH 2.2 - 2.3. The dependence of the two components on small changes in pH at pH 4.6 - 4.8 and at pH 10.1 - 10.2 also indicates this material is very sensitive to hydrogen ion concentration. It is also significant that two components were found in an otherwise one-component system with lower ionic strength at constant pH. The crystalline cucurbit globulins are therefore very sensitive to pH and ionic strength.

The origin of the two components at various pH levels is difficult to explain. The dependence of the number of components on small changes in pH and ionic strength suggests that electrostatic bonds are involved in the change from a one-component system to a two-component system.

TABLE I. Summary of Electrophoretic Differences in the Cucurbit Globulins

pH	Type of Seed Globulin: Number of globulin compounds				Number of experiments			
	Squash	Pumpkin	Cucumber	Watermelon	Squash	Pumpkin	Cucumber	Watermelon
1.0 - 1.2	-	1	-	2	-	1	-	2
2.2 - 2.3	2	2	2	2	2	1	2	2
3.9	1	-	-	1	1	3	-	1
4.0 - 4.1	-	1**	-	2*	-	1	-	2
4.2 - 4.3	1	2*	2*	2*	2	1	2	2
4.6	1	2	-	-	1	1	-	-
4.7 - 4.8	2	2	1****	1	2	2	1	1
9.1 - 9.2	2	-	2	-	2	-	2	-
9.7	-	-	-	2	-	-	-	1
10.0 - 10.1	2	2	2***	-	2	2	2	-
10.5 - 10.6	1	-	2***	-	1	-	2	-

* Only 0.01 M sodium acetate solutions indicated two components.
** Only 0.05 M sodium acetate solution was used.
*** Only 0.1 M glycine solution was used.
**** Second component found only in trace amount in 0.01 M sodium acetate.

Amino acid content of globulin from squash and pumpkin shows no difference and yet electrophoretically these two species differ slightly from each other in several ways. Cucumber and watermelon globulin differ in several ways in amino acid content but are very similar electrophoretically. Differences occur among squash, cucumber, and watermelon globulin and among pumpkin, cucumber, and watermelon globulin. As different varieties were used from those used by Smith and Green (65), the differences between their results and results found in these studies may not be significant. The findings of Thompson and Steward (74) indicate that amino acid analysis by other methods may show differences where Smith and Green failed to find any.

Sedimentation Results and Discussion

Summary of Sedimenting Components in the Cucurbit Globulins

The sedimenting components found in the seed globulins of squash, pumpkin, cucumber, and watermelon could usually be identified reasonably specifically from their sedimentation coefficients. The terminology used to distinguish the components throughout this presentation is based on the mean sedimentation coefficients obtained under specific conditions.

Components Sq^3, Pu^3, Cu^3, and Wa^3: The lightest components found had sedimentation coefficients of approximately 3 and, for each species, this is taken to be the monomer. These components were found only below the isoelectric point. Detailed results show that the sedimentation coefficients of these components vary considerably.

The following averages were obtained from analyses carried out at pH 4.3 only.

Average S°_{20} of Sq^3, Pu^3, Cu^3, Wa^3:

Sq^3: S°_{20} = 3.0. Eight analyses.

Pu^3: S°_{20} = 3.1. Seven analyses.

Cu^3: S°_{20} = 3.0. Seven analyses.

Wa^3: S°_{20} = 3.0. Nine analyses.

Components Sq^7, Pu^7, Cu^7, Wa^7: The components with sedimentation coefficients of approximately 7 appear to be dimers of Sq3, Pu^3, Cu^3, and Wa^3 respectively. The dimer was found above and below the isoelectric point, but did not have the same sedimentation coefficient in the two pH ranges. It will be shown later that below the isoelectric point the sedimentation coefficients of these components vary considerably.

Average S°_{20} of Sq^7, Pu^7, Cu^7, Wa^7:

Sq^7 at pH 4.3:	S°_{20} = 6.8.	Eight analyses.
Sq^7 above isoelectric point:	S°_{20} = 7.9.	Six analyses.
Pu^7 at pH 4.3:	S°_{20} = 6.7.	Seven analyses.
Pu^7 above isoelectric point:	S°_{20} = 8.0.	Twelve analyses.
Cu^7 at pH 4.3:	S°_{20} = 6.6.	Seven analyses.
Cu^7 above isoelectric point:	S°_{20} = 7.7.	Two analyses.
Wa^7 at pH 4.3:	S°_{20} = 6.4.	Eight analyses.
Wa^7 above isoelectric point:	S°_{20} = 8.0.	Two analyses.

Components Sq^{12}, Pu^{12}, Cu^{12}, Wa^{12}: These components with sedimentation coefficients of approximately 12 were present below and above the isoelectric point for squash globulin but they were definite only

above the isoelectric point for globulins from pumpkin, cucumber, and watermelon. Each is considered to consist of two units of the dimer Sq^7, Pu^7, Cu^7, and Wa^7, respectively. The sedimentation coefficients of these components were not as variable as those of the lighter components.

Average S^o_{20} of Sq^{12}, Pu^{12}, Cu^{12}, Wa^{12}:

Sq^{12} below the isoelectric point:	S^o_{20} = 11.8.	Four analyses.
Sq^{12} above the isoelectric point:	S^o_{20} = 12.0.	Five analyses.
Pu^{12}:	S^o_{20} = 12.7.	Twelve analyses.
Cu^{12}:	S^o_{20} = 12.2.	Seven analyses.
Wa^{12}:	S^o_{20} = 13.2.	Seven analyses.

The sedimentation coefficients are averages of a number of analyses. Variations of more than $\pm$ 10% among the individual values were uncommon. In spite of the variation in sedimentation coefficients for each component, there was good agreement between the findings of Fuerst et al. and those of the present study for squash globulin.

The variation in sedimentation coefficients between species cannot be considered significant as an insufficient number of analyses was done to be statistically analyzed. The dimer of cucumber and watermelon globulin appeared to have a consistently lower sedimentation coefficient below the isoelectric point than did either squash and pumpkin globulin.

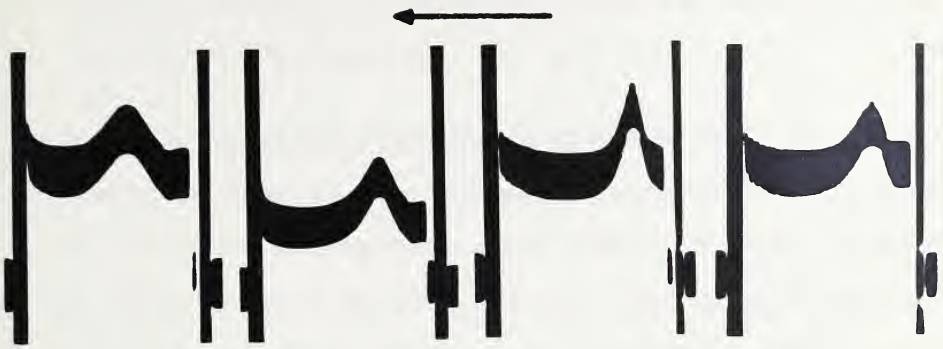
The sedimentation coefficients for the dimer were consistently higher above than below the isoelectric point. The possibility arises that the components above and below the isoelectric point are different

but this does not seem probable. Not only was the protein sedimenting in different types of ions but under different ionic strength. As electrophoretic results have shown, these proteins are highly charged molecules and thus they would be expected to exhibit a slight charge effect in the buffers of low ionic strength used below the isoelectric point. Svedberg and Pedersen (71) indicate that there is a difference of 4% in the sedimentation coefficient of the relatively low charged egg albumin determined in 0.01 M as compared with 0.1 M salt solutions.

The consistently higher value for Wa^{12} as compared with the comparable component from the other species is of doubtful significance. The measurements used for calculating this sedimentation coefficient were determined using very dilute dispersions since the solubility was low in most of the solutions studied.

Preliminary Sedimentation Results

Effect of Salt Concentration on Sedimentation Diagrams: Figure 34(A, B, C, and D) shows that globulins from all four varieties gave heterogeneous boundaries in 0.1 M sodium acetate at pH 4.3. The aggregated material had sedimentation coefficients of from $S^{o}_{20} \simeq 9$ to $S^{o}_{20} \simeq 15$. Sedimentation diagrams for 0.03 M solutions at pH 4.3 were more diffuse than diagrams of 0.01 M solutions at pH 4.3. Although protein solutions of 0.1 M glycine at approximately pH 2.2 gave sharp diagrams, proteins in higher salt concentration did not. The most satisfactory salt concentration for studying these proteins below the isoelectric point was 0.02 - 0.01 M for pH values above 3.5 and 0.04 M for pH values below pH 3.5. Above the isoelectric point, boundaries were sharp in solutions of all salt concentrations used.



A. B. C. D.

Fig. 34

A. Squash globulin in 0.1 M sodium acetate. pH 4.2.
Components $S^{\circ}_{20} \simeq$ 14 -16. Age 1 hour. Protein conc. 0.46%.

B. Pumpkin globulin in 0.1 M sodium acetate. pH 4.3.
Components $S^{\circ}_{20} \simeq$ 11. Age 1 hour. Protein conc. 0.92%.

C. Cucumber globulin in 0.1 M sodium acetate. pH 4.3.
Components $S^{\circ}_{20} \simeq$ 9. Age 1 hour. Protein conc. ?

D. Watermelon globulin in 0.1 M sodium acetate. pH 4.3.
Components $S^{\circ}_{20} \simeq$ 9. Age 1 hour. Protein conc. ?

Attention should be drawn to the electrophoretic results in high salt concentrations below the isoelectric point. While electrophoretic diagrams were sharp in 0.05 M and 0.1 M salt solutions, sedimentation diagrams appeared diffuse in these salt solutions. The diffuse sedimentation peaks could be associated with variations in the asymmetry of the molecule.

Effect of Age on the Crystals: Dispersed cucumber globulin crystals stored six months at 5° - 10°C gave sedimentation diagrams identical with those for dispersions of fresh crystals in spite of a small amount of fungal growth in the storage flask. With the other species, a storage period of six weeks had not effected the sedimentation diagrams of the dispersed crystals. The crystals under 2% mother liquor were exposed to room temperature overnight. This exposure largely destroyed their crystalline structure. Crystalline globulin of the cucurbit seeds appears to be quite stable if kept cool.

Standardization of the Ultracentrifuge: As sedimentation coefficients obtained using different instruments have differed from one ultracentrifuge to another, especially between the Spinco instrument and Svedberg's oil-turbine (73), it seemed desirable to compare results obtained with this instrument with those for others. Ovalbumin, obtained from Armour and Company, was dispersed in buffer of 0.18 ionic sodium chloride and 0.02 ionic phosphate at pH 6. A sedimentation coefficient of $S^{\circ}_{20} = 3.16$ was found as compared to values $S^{\circ}_{20} = 3.17$ and $S^{\circ}_{20} = 3.25$ reported in the literature (73). Agreement with other Spinco instruments is satisfactory but there is a significant deviation from the oil-turbine measurements (73). Sedimentation coefficients

obtained with the Spinco ultracentrifuge are about 6% lower than those obtained from the oil-turbine ultracentrifuge (57).

Effects of Fat Extraction: Although the effects of fat extraction were not extensively studied, there is no indication of any modification of the sedimentation diagrams at pH 4.3 in sodium acetate buffer by omission of fat extraction. Crystals for these analyses were prepared as previously described and one preparation of each species from which the fat extraction step had been omitted served as the test crystals.

The Effects of Age and pH Below the Isoelectric Point on Components of Globulin

Fuerst et al. (23) found the proportion of components in a dispersion altered with age. Since this could be used as a simple test for association-dissociation phenomena, dispersions of globulin from the four species were analyzed in the centrifuge at intervals of time after dispersion.

Throughout this work many of the individual analyses were repeated and gave reasonably consistent results. It has been pointed out that sedimentation analyses carried out under similar conditions showed some variation in sedimentation coefficients. Results, such as proportion of components, number of components and diffuseness of boundaries were always consistent with those done under similar conditions.

Squash Seed Globulin: When squash globulin was dispersed in 0.01 M sodium acetate, pH 4.3, three components appeared. Figure 35 (A,B,C,D,E)

A. Sedimentation analysis of squash globulin in 0.01 M sodium acetate. pH 4.3.
Components: Sq^3, Sq^7, Sq^{12}. Age 1/2 hour. Protein conc. 0.81%.
Photographs at 16 min. intervals.

B. Squash globulin in 0.01 M sodium acetate. pH 4.3.
Components: Sq^3, Sq^7, Sq^{12}. Age 1 1/2 hours. Protein conc. 0.72%.

C. Same dispersion as A.
Components: Sq^3, Sq^7, Sq^{12}. Age 2 hours.

D. Same dispersion as A.
Components: Sq^3, Sq^7, Sq^{12}. Age 12 hours.

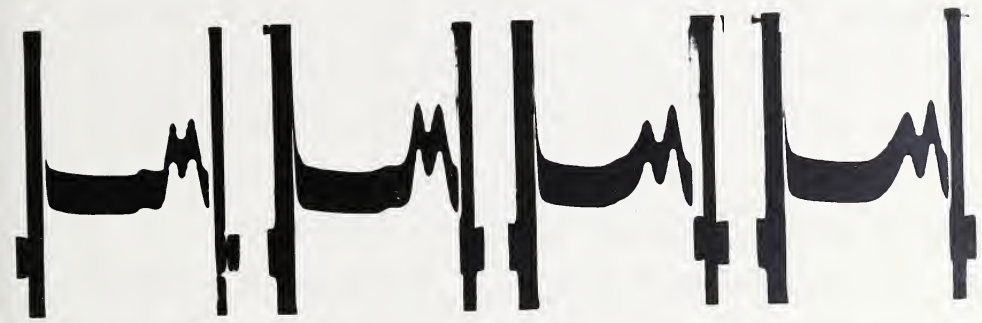
E. Same dispersion as A.
Components: Sq^3, Sq^7. Age 19 hours.

F. Squash globulin in 0.01 M sodium acetate. pH 3.6.
Component: Sq^3. Age 2 hours. Protein conc. 0.65%.

G. Same dispersion as F.
Components: Sq^3, Sq^7. Age 1 day.

Fig. 35

A



B C D E

F G

illustrates the alteration in concentration of these components with time. A sedimentation analysis was made one-half hour after dispersal of the globulin and the result is presented in Fig. 35A. Both Sq^7 and Sq^{12} decreased in concentration with time while Sq^3 increased. Soon after the disappearance of Sq^{12}, Sq^7 begins to increase in concentration. Fuerst et al. found that component Sq^7 continued to increase with age and indicated that the Sq^7 formed with age may differ from the Sq^7 formed on dispersion. This is compatible with the data found here.

Fuerst et al. did not find Sq^{12} in dispersions below the isoelectric point, but as there was only a trace of this component which disappeared in a few hours, it seems probable that it represents material that was incompletely dispersed in the freshly prepared dispersions.

When squash globulin was dispersed in 0.01 M sodium acetate buffer at pH 3.6, two components appeared only in the one-day-old dispersions as Fig. 35F and G show. The two-hour-old dispersion was definitely not homogeneous as the skewed peak indicates, but the two components did not separate.

Figure 36 (A, B, C) shows the alteration of sedimentation diagrams with age in 0.03 M glycine buffer at pH 2.7. Again, as shown in the acetate buffer, the two-hour-old dispersion formed one skewed peak on sedimentation, but at 25 days two distinct symmetrical peaks had separated. An apparent increase in sedimentation coefficient of component Sq^7 is associated with a complete separation of the two peaks. There is no obvious explanation for the increase in sedimentation coefficient but a decrease in the frictional coefficient would be compatible with the data.

Fig. 36

A. Squash globulin in 0.03 M glycine. pH 2.7.
Components: Sq^3, Sq^7? Age 2 hours. Protein conc. 1.1%.

B. Same dispersion as A.
Components: Sq^3, Sq^7. Age 3 days.

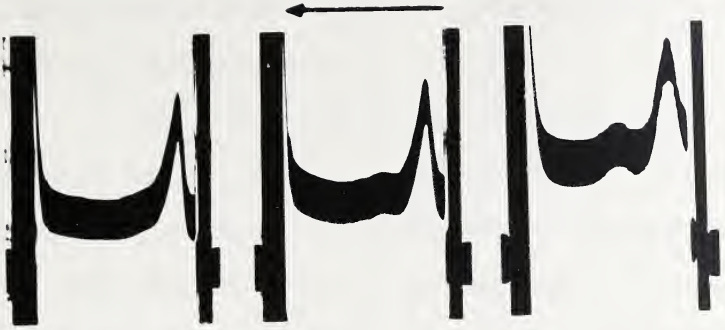
C. Same dispersion as A.
Components: Sq^3, Sq^7. Age 25 days.

D. Squash globulin in 0.01 M glycine. pH 1.4.
Components: Sq^3, Sq^7. Age 2 hours. Protein conc. 0.90%.

E. Same dispersion as D.
Components: Sq^3, Sq^7. Age 2 days.

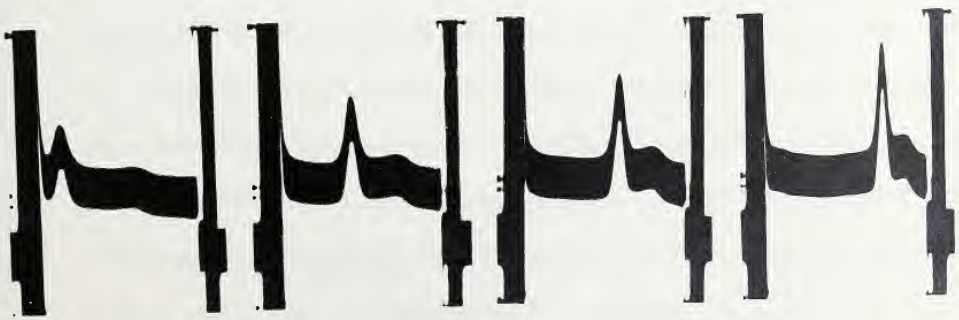
F. Sedimentation run of pumpkin globulin in 0.01 M sodium acetate. pH 4.3.
Components: Pu^3, Pu^7. Age 1/2 hour. Protein conc. 0.69%. Photographs at 16 min. intervals. Last photograph at 32 min. interval.

Fig. 36



A B C

D E



F

One sedimentation analysis of squash globulin in 0.003 M sodium phosphate buffer at pH 2.2 indicates results similar to those found in 0.03 M glycine buffer at pH 2.7.

When squash globulin was dispersed in 0.01 M glycine buffer at the very low pH of 1.4 (Fig. 36D, E) two components appeared, probably Sq^3 and Sq^7. The sedimentation coefficients were higher than those obtained at pH 2.7 and there was a much more definite separation of the components. The change in sedimentation coefficients is discussed later.

The preceding results can be explained satisfactorily only on the basis that crystalline squash seed globulin forms an association-dissociation system on dispersal. Although association appears to predominate, dissociation did occur.

Pumpkin Seed Globulin: Dispersions of pumpkin globulin in 0.01 M sodium acetate at pH 4.3 contained at least two components, Pu^3 and Pu^7 which, with age, alter in relative concentration.

Figure 36F presents the results for the sedimentation run one-half hour after preparation. The obvious explanation for the plateau region between the peaks is that the slower sedimenting material was arising from the more rapidly sedimenting peak. This is probably what occurred as the more rapidly sedimenting peak was decreasing in area while the more slowly sedimenting peak and plateau region were increasing in area. Figure 37 (A,B,C,D) shows a progressive increase in the concentration of component Pu^3 and a decrease in the concentration of component Pu^7 with age.

In 0.01 M sodium acetate buffer at pH 3.6, pumpkin globulin dissociates into two components within one day after

Fig. 37

A. Pumpkin globulin in 0.01 M sodium acetate. pH 4.3.
Same dispersion as Fig. 36F.
Components: Pu^3, Pu^7. Age 3 hours. Protein conc. 0.69%.

B. Same dispersion as Fig. 36F.
Components: Pu^3, Pu^7. Age 9 hours.

C. Same dispersion as Fig. 36F.
Components: Pu^3, Pu^7. Age 2 days.

D. Same dispersion as Fig. 36F.
Components: Pu^3, Pu^7. Age 7 days.

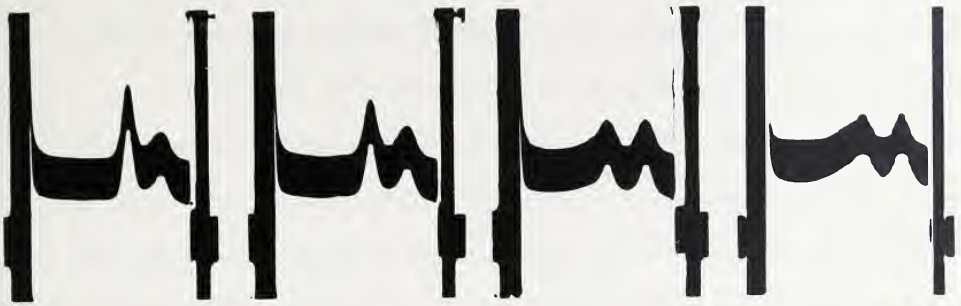
E. Pumpkin globulin in 0.01 M sodium acetate. pH 3.6.
Components: Pu^3, Pu^7? Age 3 1/2 hours. Protein conc. 0.48%.

F. Same dispersion as E.
Components: Pu^3, Pu^7. Age 2 days.

G. Pumpkin globulin in 0.03 M glycine. pH 2.6.
Components: Pu^3, Pu^7? Age 5 hours. Protein conc. 0.93%.

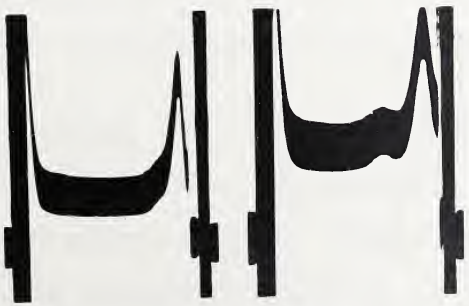
H. Same dispersion as G.
Components: Pu^3, Pu^7. Age 23 days.

Fig. 37



A B C D

E F



G H

preparation, probably Pu^3 and Pu^7. Figure 37E, F shows the increase in concentration of the heavier component, Pu^7. The results are very similar to those for squash globulin at this pH.

Figure 37G, H shows the effect of age on pumpkin globulin dispersed for five hours and 23 days in 0.03 M glycine buffer at pH 2.6. The five-hour-old dispersion showed only a trace of component Pu^7 but as the second diagram shows, Pu^7 increased slightly in concentration. The peak of component Pu^3 is symmetrical in the 23-day dispersion but was strongly skewed to the left in a five-hour-old dispersion. It is evident that the results are similar to those found in squash globulin at pH 2.7.

Figure 38A, B shows some difference between a three-hour-old dispersion and a two-day-old dispersion of pumpkin globulin in 0.01 M glycine buffer at pH 1.4. Nevertheless, component Pu^3 appears to be associating slowly to Pu^7. The rate of association seems to be greater at pH 1.4 during the first three hours after dispersion at this pH than at pH 2.6 and pH 3.6. This would be expected as the ionic strength was higher at pH 1.4.

The results from the aging experiments on crystalline pumpkin seed globulin strongly indicate that on dispersal the globulin forms an association-dissociation system.

Cucumber Seed Globulin: When cucumber globulin was dispersed in 0.01 M sodium acetate, pH 4.3, two components, Cu^3 and Cu^7, were observed on sedimentation analysis. Figure 38C shows a sedimentation run started within one-half hour after dispersal of the globulin in buffer at pH 4.3. The material in the plateau region between the two peaks appears to be arising from the slower sedimenting component

Fig. 38

A. Pumpkin globulin in 0.01 M glycine. pH 1.4.
Components: Pu^3, Pu^7. Age 3 hours. Protein conc. 0.76%.

B. Same dispersion as A.
Components: Pu^3, Pu^7. Age 2 days.

C. One sedimentation analysis of cucumber globulin in 0.01 M sodium acetate. pH 4.3.
Components: Cu^3, Cu^7. Age 1/2 hour. Protein conc. 0.70%.
Photographs at 16 min. intervals.

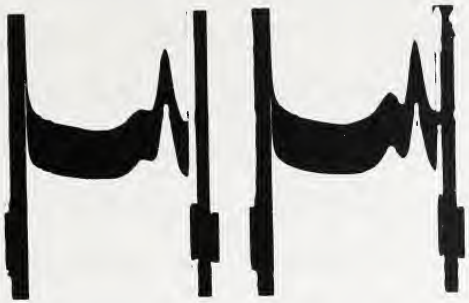
D. Cucumber globulin in 0.01 M sodium acetate. pH 4.3.
Components: Cu^3, Cu^7. Age 3 hours. Protein conc. 0.71%.

E. Cucumber globulin in 0.01 M sodium acetate. pH 4.3.
Components: Cu^3, Cu^7. Age 1 day. Protein conc. 0.82%.

F. Same dispersion as C.
Components: Cu^3, Cu^7. Age 6 days.

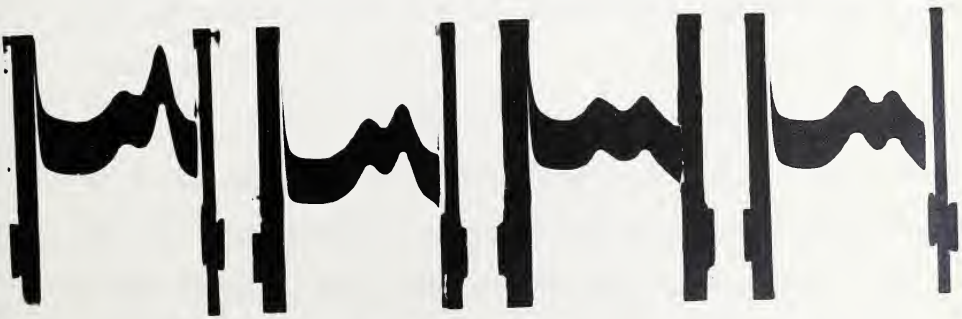
G. Same dispersion as D.
Components: Cu^3, Cu^7. Age 9 days.

Fig. 38



A B

C



D E F G

as changes in the relative areas of the peaks would indicate. Therefore, the tendency of the reaction was probably toward association. It is evident that Cu^3 was either produced immediately on dispersion or that dissociation took place very rapidly.

Figure 38 (D, E, F, G) shows that the slower sedimenting component, Cu^3, decreased in concentration while the more rapidly sedimenting component, Cu^7, increased in concentration for a period of at least nine days.

Dispersions of cucumber globulin in 0.01 M sodium acetate buffer at pH 3.6 contained two poorly resolved components after one day, probably Cu^3 and Cu^7. Six-hour-dispersions were definitely not homogeneous by sedimentation analysis as the peak was slightly skewed to the left, Fig. 39 (A, B). Association occurs more slowly at this pH. Since the diagrams seem to indicate results similar to those for squash globulin at pH 3.6, no further discussion seems warranted.

Figure 39 (C, D) shows only a strongly skewed peak in 0.01 M glycine buffer at pH 3.0 even six days after dispersal. It was thought that these results strongly indicate that low pH and low salt concentration, and not necessarily a specific salt, are conducive to dissociation.

Results of globulin dispersed in 0.04 M glycine buffer at pH 2.3 were similar to those found in 0.01 M glycine buffer at pH 3.0. Dispersions of cucumber globulin in 0.04 M sodium formate buffer at pH 2.3 gave sedimentation diagrams similar to those found in glycine buffer at pH 2.2.

Fig. 39

A. Cucumber globulin dispersed in 0.01 M sodium acetate. pH 3.6.
Component: Cu^3. Age 6 hours. Protein conc. 0.70%.

B. Same dispersion as A.
Components: Cu^3, Cu^7? Age 2 days.

C. Cucumber globulin in 0.01 M glycine. pH 3.0.
Component: Cu^3. Age 1 day. Protein conc. 0.61%.

D. Same dispersion as C.
Component: Cu^3. Age 6 days.

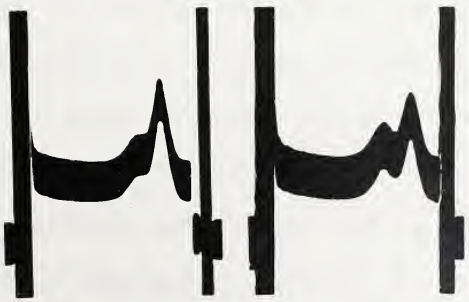
E. Cucumber globulin in 0.01 M glycine. pH 1.4.
Components: Cu^3, Cu^7. Age 7 hours. Protein conc. 0.1%.

F. Same dispersion as E.
Components: Cu^3, Cu^7. Age 2 days.

Fig. 39

A B

C D



E F

Figure 39 (E, F) shows two components in 0.01 M glycine buffer at pH 1.4. Obviously, these formed more rapidly than at pH 3.0 and pH 3.6. The association was quite slow as the difference between seven-hour-old and two-day-old dispersions, although distinct, was small. The increase in sedimentation coefficients of components that occurred at pH 1.4 will be discussed later.

It has been shown that association occurs in the cucumber seed globulin below the isoelectric point. Although no evidence has been presented that indicates dissociation, sedimentation runs above the isoelectric point show that dissociation must have occurred in this protein. Therefore, it is concluded that cucumber seed globulin probably is an association-dissociation system.

Watermelon Seed Globulin: Watermelon globulin, when dispersed in 0.01 M sodium acetate at pH 4.3, appears as one strongly skewed peak when analyzed in the ultracentrifuge one-half hour after dispersal, but two sedimenting peaks, Wa^3 and Wa^7, appear within two hours after dispersal. Figure 40 (A) represents a sedimentation run started within one-half hour after dispersal. There is no way of knowing whether or not the globulin dissociates into component Wa^3 immediately on dispersal or whether the component was in the form of Wa^3 before dispersal. It seems probable that the globulin was initially in an associated form. Regardless of which phenomenon occurred, there is a continual increase in concentration of the more rapidly sedimenting component from one-half hour after dispersal to eight days after dispersal as Figure 40 (B, C, D, E) shows.

Dispersions of watermelon globulin at pH 4.3 indicated

Fig. 40

A. One sedimentation analysis of watermelon globulin in 0.01 M sodium acetate. pH 4.3.
Components: Wa^3. Age 1/2 hour. Protein conc. 0.63%.
Photographs at 16 min. intervals. Last photograph 32 min. interval.

B. Same dispersion as A.
Components: Wa^3, $S^{\circ}_{20} \simeq 4.5?$, Wa^7. Age 2 hours.

C. Same dispersion as A.
Components: Wa^3, $S^{\circ}_{20} \simeq 4.5?$, Wa^7. Age 10 hours.

D. Same dispersion as A.
Components: Wa^3, Wa^7. Age 8 days.

E. Same dispersion as A and same sedimentation analysis as B.
Components: Wa^3, $S^{\circ}_{20} \simeq 4.5?$, Wa^7.
Photograph taken 16 mins. after the photograph taken in B.

F. Watermelon globulin in 0.01 M sodium acetate. pH 3.6.
Component: Wa^3. Age 6 hours. Protein conc. 0.64%.

G. Same dispersion as F.
Components: Wa^3, Wa^7. Age 2 days.

H. Watermelon globulin in 0.01 M glycine. pH 1.4.
Components: Wa^3, Wa^7. Age 1 day. Protein conc. 0.88%.

I. Same dispersion as H.
Components: Wa^3, Wa^7. Age 4 days.

Fig. 40

A

B C D E

F G H I

a third component sedimenting between Wa^3 and Wa^7 with $S^{\circ}_{20} \simeq 4.5$, Fig. 40 (E). The hint of this component had disappeared in the eight-day-old dispersion, Fig. 40 (D). As the diagrams were very diffuse, it would be difficult to attribute the phenomena to another component, but since this phenomena occurred in several dispersions at pH 4.3, it cannot be completely ignored. The only explanation that can be offered is that Wa^3 may form an unstable more or less symmetrical variant molecule which could be expected to sediment more rapidly than Wa^3. As this phenomenon disappeared in older dispersions, these molecules could have changed to the apparently more stable and probably less symmetrical Wa^3.

Although watermelon globulin is not homogeneous as indicated by sedimentation analyses in 0.01 M sodium acetate buffer at pH 3.6 (Fig. 40, F, G), the more rapidly sedimenting component, supposedly Wa^7, formed very slowly and was poorly resolved on sedimentation analysis even in two-day-old dispersions.

One analysis on a one-day-old dispersion of watermelon globulin in 0.01 M glycine buffer at pH 2.9 showed results very similar to the results found at pH 3.6.

Sedimentation analyses in 0.01 M glycine at pH 1.4, Fig. 40 (H, I), show two components in one and four-day-old globulin dispersions. Although the more rapidly sedimenting component became more sharply defined with age, there appears to be no evidence of either component changing in concentration during this period. But it seems probable that either association or dissociation occurred to the extent shown in Fig. 40 (H, I).

There is no real evidence that dissociation occurred below the isoelectric point but from studies made above the isoelectric point to be presented later, dissociation must have occurred in watermelon seed globulin. Since association of this protein has been shown to occur, it is concluded that watermelon seed globulin probably forms an association-dissociation system.

Interpretation of Aging Results: The conclusion that the globulins from the four species probably form association-dissociation systems is not meant to infer that all the processes of association and dissociation are reversible. In all probability, they are not in some cases. This would be especially true of the association process at pH values below the isoelectric point. It seems possible that the components $S^{\circ}_{20} \simeq 7$ were in some cases modified dimers or denatured dimers. If this is so, it can be seen that there would be difficulties in attempting to apply reaction kinetics to the cucurbit globulin systems.

The most logical interpretation of the results presented in the preceding pages is that seed globulins from the four cucurbit species form association-dissociation systems.

Effects of pH on Sedimentation Coefficients

The effect of pH on the cucurbit globulins has been presented in previous sections but nothing was said of the variation in sedimentation coefficients that occurred. The analyses in the previous sections were used in this section for study of this variation.

Below the isoelectric point, a decrease in sedimentation coefficients paralleled a decrease in pH. As this occurred in all four species, the results are presented and discussed together. Dispersions discussed in this section all contained the monomer of the corresponding globulin and probably the dimer.

Analyses carried out at pH 2.7 gave sedimentation coefficients from one-half to one-third of the value found at pH 4.3. Although dissociation of the monomer was suspected, Fig. 41 (A, B) and Fig. 42 (A, B) show a gradual decrease in sedimentation coefficients with a decrease in pH rather than any abrupt change. This would suggest that the change was probably due to modification of the monomer and not to dissociation.

The electrophoretic results showed a decrease in mobility with a decrease in pH below the isoelectric point. It is questionable whether or not the decrease in sedimentation coefficients and mobilities are the product of similar protein modifications. No similar decrease in sedimentation coefficients occurred with an increase in pH above the isoelectric point while electrophoretic mobilities did decrease at pH values above 9. Further experimentation may show a relationship between electrophoretic and sedimentation results below the isoelectric point but the present information does not show any obvious relationship.

The decrease in the sedimentation coefficients cannot be attributed to a charge effect as electrophoretic analyses in solutions of identical salt concentration and pH show that the charge on the molecule

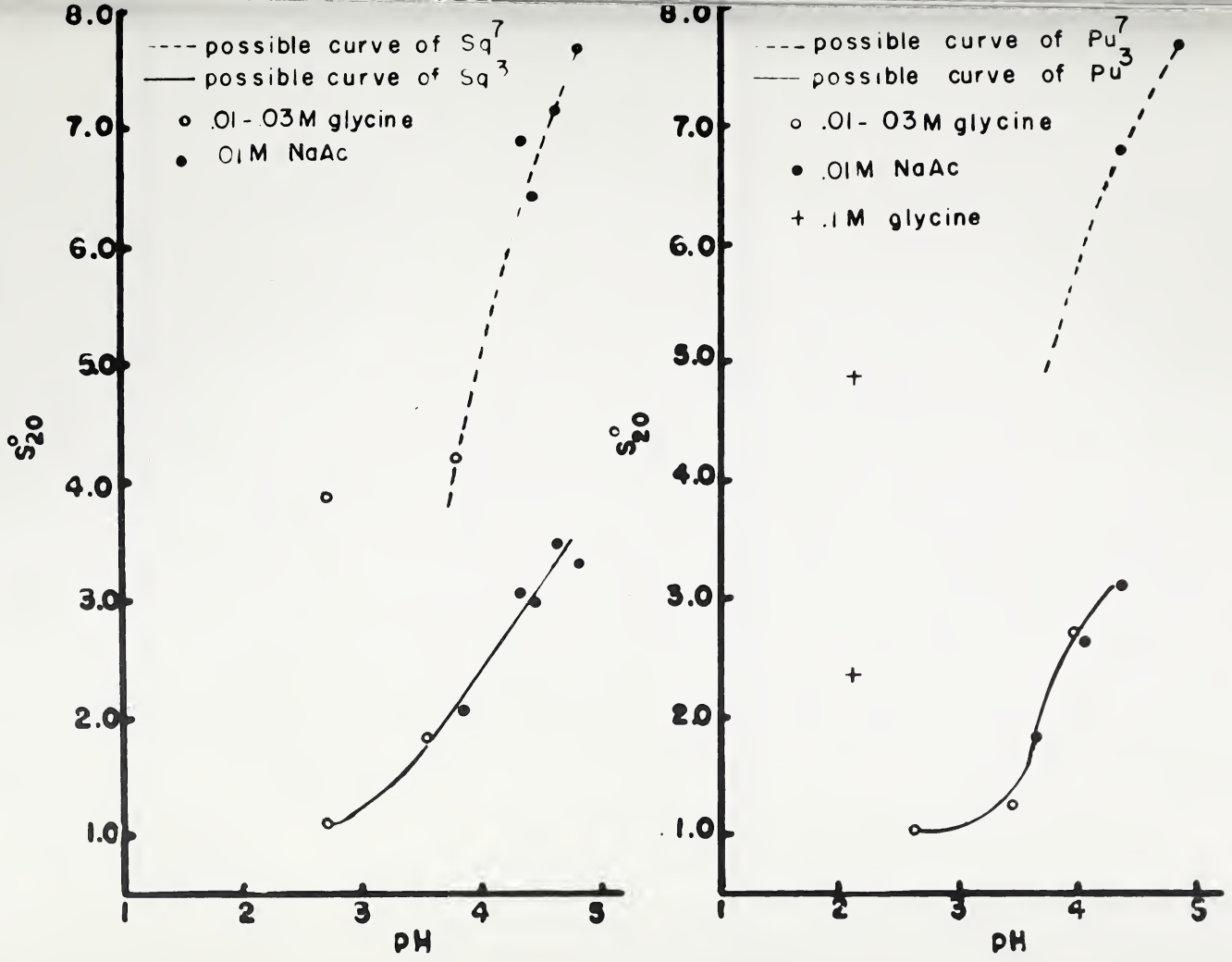


Fig. 41

Effect of pH on sedimentation coefficients of squash and pumpkin globulin.

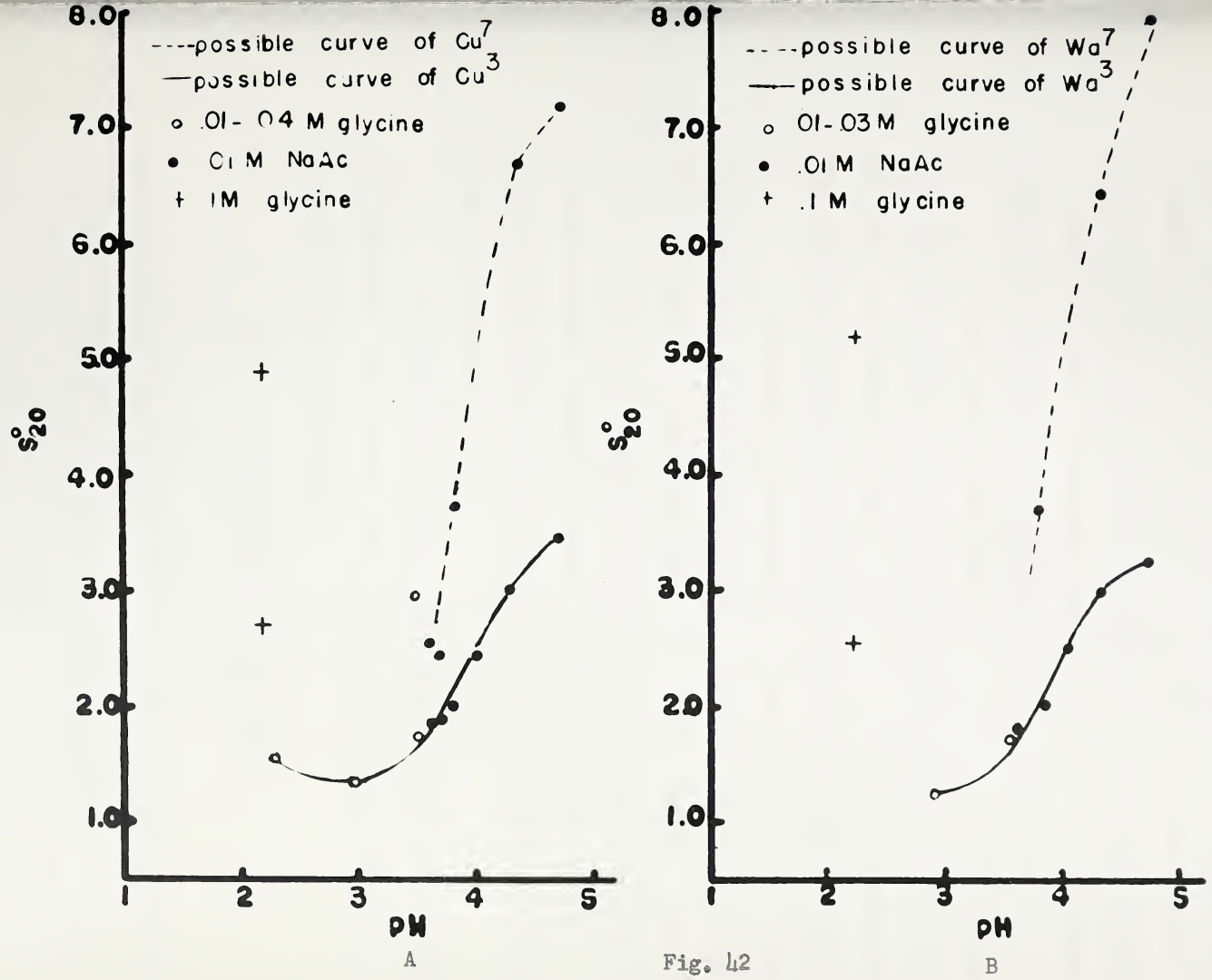


Fig. 42

Effect of pH on sedimentation coefficients of cucumber and watermelon globulin.

apparently decreased with a decrease in pH. Although the electrophoretic results are strictly comparable only with three-day-old dispersions, it seems unlikely that any change in charge occurred during the three days required for dialysis. In any case, analyses at slightly higher pH values gave similar results, for the lighter component, with two-hour and three-day-old dispersions.

Additional evidence that the reduction in sedimentation coefficients is not a charge effect is obtained with the dispersions in higher salt concentrations at pH 2 - 3. These give sedimentation coefficients of approximately one-half that of the monomer at pH 4.3.

A decrease in sedimentation coefficients with a decrease in pH has been found in bovine, human, and horse serum albumin and to lesser extent in ovalbumin (16). The authors explain the variation as the result of an expansion or extension of the molecules and cite increased partial specific volumes at low pH values as evidence. Evidently, the exact nature of this molecular alteration is still a matter of speculation.

To explain the reduced sedimentation value by an increase in frictional coefficients, large changes in asymmetry would have to occur. Using the equation

$$S_{20}^{o} = \frac{1.2 \times 10^{-15} \, Me^{2/3} (1 - \bar{v}\rho)}{f/fo \qquad \bar{v}\, 1/3}$$

given by Svedberg and Pedersen (71), it can be calculated that the frictional coefficient would have to change by a factor of 2 or 3 to explain the large decreases found. The partial specific volume would probably vary at the same time as molecular configuration.

A small variation in partial specific volume would reflect a large variation in sedimentation coefficients.

Large frictional coefficients would be expected to reflect concentration-dependent sedimentation in the dispersions. An attempt was made to show this dependence with cucumber globulin at pH 3.6, watermelon globulin at pH 3.8 and squash globulin at pH 3.8. Only for cucumber globulin dispersions at pH 3.6 did sedimentation coefficients give any indication of being concentration-dependent. The evidence indicates that the decrease in sedimentation coefficients is due to molecule extension or expansion.

The sedimentation diagrams already presented show that the degree of association is small below pH 4.0. This would indicate that these globulins tend toward dissociation at low pH values. Numerous examples of dissociation favoured by low pH can be found in the literature. The work of Fredricq and Neurath (21) and Johnson (35) will serve as examples. As the literature already reviewed indicates, dissociation at low pH values is usually attributed to mutual repulsion (60, 83) of the more highly charged protein molecules. Cucurbit globulins have been shown, however, to decrease in effective charge as the pH is lowered. Therefore, it is difficult to explain the highly dissociated state at low pH values by mutual repulsion of the charged protein

molecules. A more logical hypothesis to explain the highly dissociated state at low pH values would be one involving changes in configuration. If the usual binding sites for association were masked, or less available due to configuration changes, one would expect association to be retarded. Although there is only an indication of the nature of the binding sites, it does not seem premature to suggest that they may be less available in the globulin at low pH values. Research into the nature of the binding sites in these globulins may prove rewarding.

The large variation in sedimentation coefficients found in the cucurbit seed globulins could be explained if changes in symmetry of the molecules occurred easily. Thus, the variation could be due either to a change in frictional coefficient, a change in partial specific volume, or to inconsistencies in the rates of association-dissociation due to a labile characteristic of the binding sites. The electrophoretic results already discussed have also indicated a rearrangement of the protein molecule.

The Effects of pH Near and Above the Isoelectric Point

Near the Isoelectric Point: In addition to the results which have already been presented on the effect of pH and aging, some experiments were done on the effect of pH between pH 4.3 and the isoelectric point.

When squash globulin was dispersed in 0.01 M sodium acetate, pH 4.4, and dialyzed overnight to pH 4.8, association occurred to Sq^7 and Sq^{12} to the extent that only a trace of the dissociated product, Sq^3, was present, Fig. 43 (A). There was a

Fig. 43

A. Squash globulin in 0.01 M sodium acetate. pH 4.8.
Components: Sq^3?, Sq^7, Sq^{12}. Age 1 hour. Protein conc. 0.73%.
Indistinct peaks due to faulty original sedimentation plate.

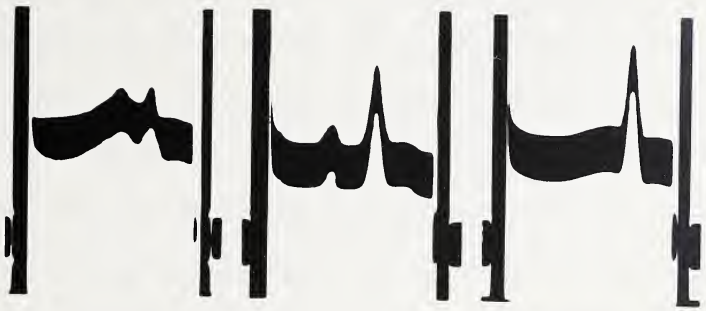
B. Squash globulin in 0.01 M sodium acetate. pH 4.6.
Components: Sq^3?, Sq^7, Sq^{12}. Age 2 hours. Protein conc. 0.69%.

C. Pumpkin globulin in 0.01 M sodium acetate. pH 4.8.
Components: Pu^7, $S^o_{20} \simeq 12$. Age 1 day. Protein conc. 0.63%.

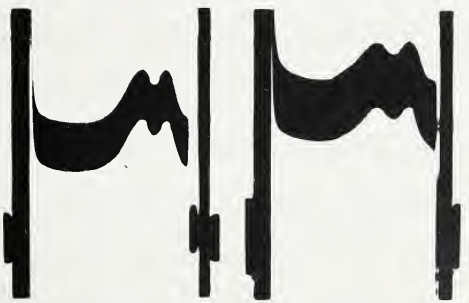
D. Cucumber globulin in 0.01 M sodium acetate. pH 4.7.
Components: Cu^3, Cu^7. Age 7 hours. Protein conc. 0.82%.

E. Watermelon globulin in 0.01 M sodium acetate. pH 4.7.
Components: Wa^3, Wa^7. Age 6 hours. Protein conc. 0.50%.

Fig. 43



A B C



D E

marked increase in the concentration of component Sq^{12} and the skewed peak of Sq^{12} indicates that more rapidly sedimenting material was also present.

It will be recalled that Fuerst et al. (23) found only component Sq^{7} and more rapidly sedimenting non-specific aggregates at pH 4.8. As their methods of dispersion were different from those used here, this discrepancy is not thought to be serious.

Figure 43 (B) shows the results of 0.01 M sodium acetate buffer at pH 4.6 on squash globulin one hour after dispersion. Of the three components present, component Sq^{7} is dominant, but as Sq^{12} is more prominent than Sq^{3} the tendency appears to be toward association at this higher pH.

Pumpkin globulin was dispersed in 0.01 M sodium acetate at pH 4.8 by the same method used for the squash globulin at this pH. It appears as one sharply defined peak (Pu^{7}) and one very diffuse peak, Fig. 43 (C). There appears to be a tendency towards association at this pH.

When cucumber globulin was dispersed in 0.01 M sodium acetate pH 4.7 in the usual way and analyzed in the ultracentrifuge, two sedimenting peaks appeared (Cu^{3} and Cu^{7}). Figure 43 (D) shows that the tendency toward association was greater at pH 4.7 than at lower pH values.

Figure 43 (E) presents a sedimentation diagram for watermelon globulin dispersed in 0.01 M sodium acetate buffer at pH 4.7. While no Wa^{12} appears in this diagram, there is a slight shift toward association.

Above the Isoelectric Point: Dispersions of the four cucurbit seed globulins in 10% (1.7 M) sodium chloride at pH 5.8 - 6.0 gave very similar sedimentation results. Figure 44 (A, B, C, D) presents these sedimentation diagrams for squash, pumpkin, cucumber, and watermelon seed globulin. The main component obviously contained most of the protein while traces of a slower and a more rapidly sedimenting component were evident in all but one diagram. In dispersions of watermelon globulin, the slower sedimenting component was absent. As the sedimentation coefficient of the main peak was 10.4 to 11.0, it possibly represents two units of Sq^7, Pu^7, Cu^7, and Wa^7. As the sedimentation coefficients of the two trace components were $S^\circ_{20} \doteq 7$ and $S^\circ_{20} \doteq 16$, they were probably the dimer of component Sq^3, Pu^3, Cu^3, and Wa^3 and an association product of undetermined origin. Although the sedimentation coefficients of the components were different from those found in any other preparation, it seems reasonable to conclude that they represent basically the same units studied in the dispersions that have already been discussed. The atmosphere of different buffers could be expected to cause slight modification in the molecule which could modify its sedimentation characteristics.

At pH 6.2, 6.5, 7.0, 7.4, 8.0, 8.5, all dispersions of pumpkin globulin in 1.0 M sodium chloride gave very similar results.

Figure 44 (E, F, G, H) shows the effects of increasing pH on day-old squash globulin dispersions in 0.1 M glycine buffer (pH 8.2 and 10.7). The results for the first three were generally similar with the predominant component being Sq^{12}. At pH 10.7, however, the globulin was almost completely in the form of Sq^7.

Fig. 44

A. Squash globulin in 10% (1.7 M) sodium chloride. pH 6.0.
Components: Sq^7?, Sq^{12}, $S^{\circ}_{20} \simeq 16$. Age 1 day. Protein conc. 0.62%.

B. Pumpkin globulin in 10% (1.7 M) sodium chloride. pH 5.9.
Components: Pu^7?, Pu^{12}, $S^{\circ}_{20} \simeq 16$. Age 1 day. Protein conc. 0.59%.

C. Cucumber globulin in 10% (1.7 M) sodium chloride. pH 6.0.
Components: Cu^7?, Cu^{12}, $S^{\circ}_{20} \simeq 15$. Age 1 hour. Protein conc. 0.64%.

D. Watermelon globulin in 10% (1.7 M) sodium chloride. pH 5.8.
Components: Wa^{12}, $S^{\circ}_{20} \simeq 16$. Age 1 day. Protein conc. 0.61%.

E. Squash globulin in 0.1 M glycine. pH 8.2.
Components: Sq^7, Sq^{12}. Age 1 day. Protein conc. 0.60%.

F. Squash globulin in 0.1 M glycine. pH 8.8.
Components: Sq^7, Sq^{12}. Age 1 day. Protein conc. 0.52%.

G. Squash globulin in 0.1 M glycine. pH 9.3.
Components: Sq^7, Sq^{12}. Age 1 day. Protein conc. 0.59%.

H. Squash globulin in 0.1 M glycine. pH 10.7.
Components: Sq^7, Sq^{12}? Age 1 day. Protein conc. 0.74%.

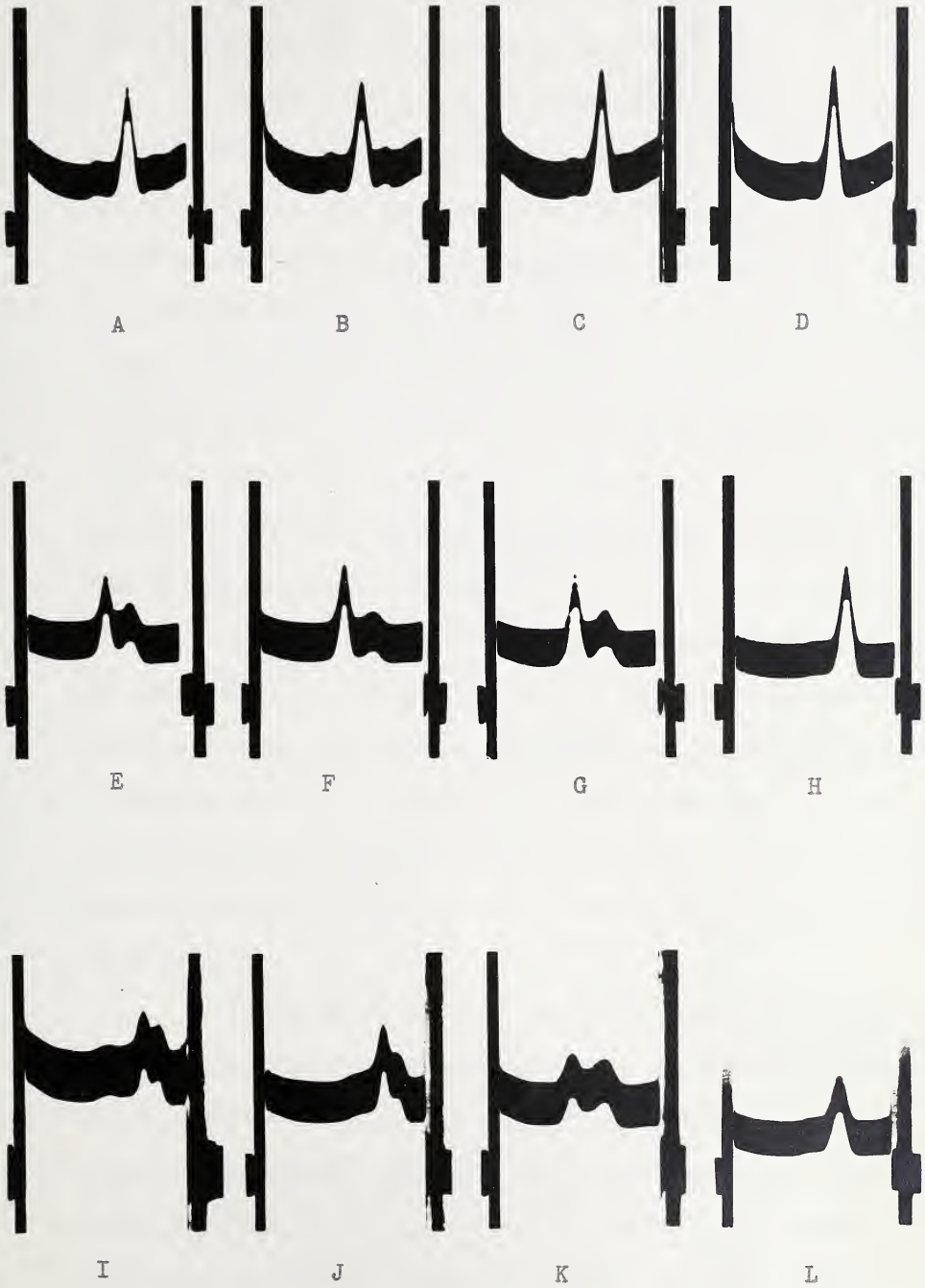
I. Pumpkin globulin in 0.1 M glycine. pH 8.2.
Components: Pu^7, Pu^{12}, $S^{\circ}_{20} \simeq 20$. Age 1 day. Protein conc. 0.33%.

J. Pumpkin globulin in 0.1 M glycine. pH 8.8.
Components: Pu^7, Pu^{12}. Age 1 day. Protein conc. 0.49%.

K. Pumpkin globulin in 0.1 M glycine. pH 9.3.
Components: Pu^7, Pu^{12}. Age 1 day. Protein conc. 0.49%.

L. Pumpkin globulin in 0.1 M glycine. pH 10.7.
Components: Pu^7, Pu^{12}? Age 1 day. Protein conc. 0.56%.

Fig. 44



The corresponding analyses of pumpkin seed globulin gave similar results, Fig. 44 (I, J, K, L). At pH 8.2, however, there was a trace of a more rapidly sedimenting component.

Figure 45 (A, B, C, D) shows the effect of increasing pH on day-old cucumber globulin dispersions in 0.1 M glycine buffers (pH 8.2 - 11.1). The protein in the dispersion was in the form of component Cu^{12} until at pH 11.1 a second slower sedimenting component was found. The third diagram in this series does not agree with the others. The component appears to be Cu^{7}, but there is no logical explanation for this result. In the following discussion it has been ignored. It is planned to repeat the analyses. It is not known whether the traces of slower and more rapidly sedimenting components in Fig. 45 (B) have any significance.

Although it could be argued that the slower component Cu^{7} was only slightly soluble below pH 11.1, it seems very unlikely that this would explain its sudden appearance. If solubility caused Cu^{7} to appear, it would be expected that the amount of Cu^{7} in the dispersions would increase as the pH increased. It seems much more likely that the appearance of Cu^{7} is related to dissociation of Cu^{12} at pH of 11.1.

Figure 45 (E, F, G, H) shows the effect of increasing pH on day-old watermelon globulin dispersions in 0.1 M glycine buffers (pH 8.8 - 11.1). Only Wa^{12} was present below pH 10.7, but at pH 10.7 and pH 11.1 component Wa^{7} appeared. A globulin dispersion in 0.1 M sodium chloride at pH 8.8 gave results similar to dispersions in glycine at the same pH. Only further analyses would indicate whether

Fig. 45

A. Cucumber globulin in 0.1 M glycine. pH 8.2.
Components: Cu^{12}. Age 1 day. Protein conc. 0.63%.

B. Cucumber globulin in 0.1 M glycine. pH 9.3.
Components: Cu^{7}(trace), Cu^{12}, $S^{o}_{20} \simeq 19$ (trace). Age 1 day.
Protein conc. 0.68%.

C. Cucumber globulin in 0.1 M glycine. pH 10.7.
Components: Cu^{7}. Age 14 hours. Protein conc. 0.71%.

D. Cucumber globulin in 0.1 M glycine. pH 11.1.
Components: Cu^{7}, Cu^{12}. Age 1 day. Protein conc. 0.95%.

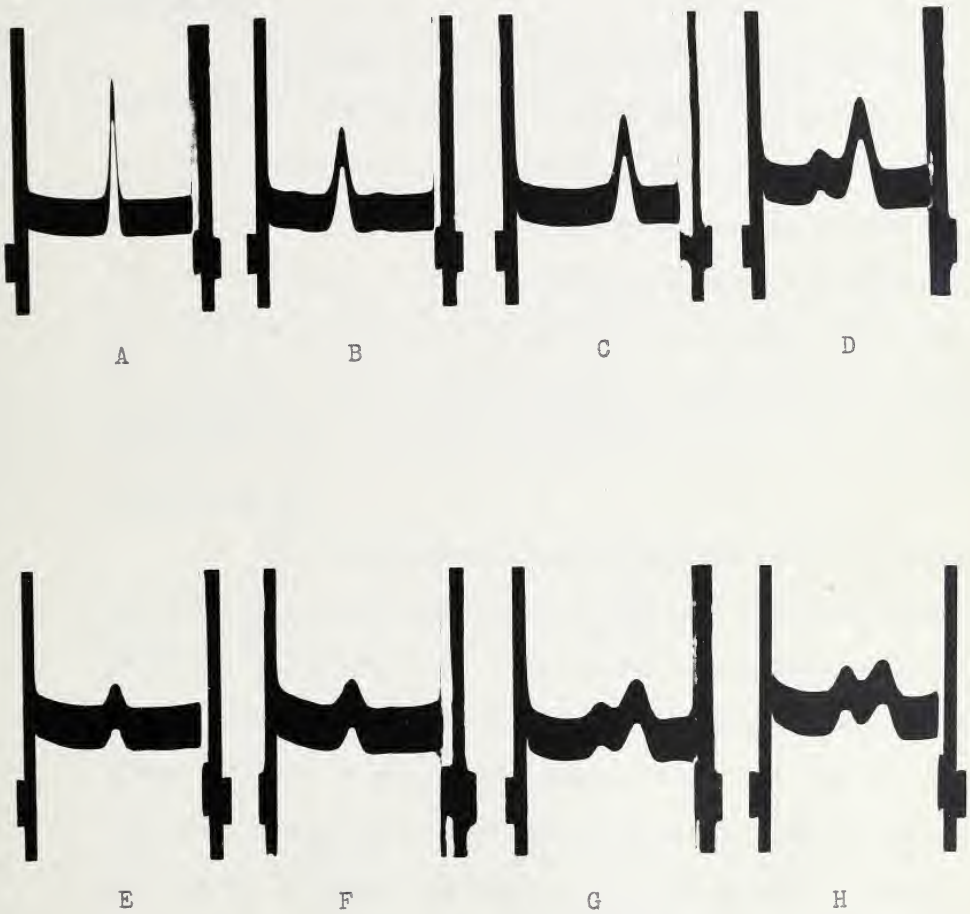
E. Watermelon globulin in 0.1 M glycine. pH 8.8.
Components: Wa^{12}. Age 1 day. Protein conc. 0.18%.

F. Watermelon globulin in 0.1 M glycine. pH 9.3.
Components: Wa^{12}. Age 1 day. Protein conc. 0.20%.

G. Watermelon globulin in 0.1 M glycine. pH 10.7.
Components: Wa^{7}, Wa^{12}. Age 1 day. Protein conc. 0.64%.

H. Watermelon globulin in 0.1 M glycine. pH 11.0.
Components: Wa^{7}, Wa^{12}. Age 1 day. Protein conc. 0.5%.

Fig. 45



or not the appearance of Wa^7 was due to solubility factors but it seems possible that dissociation took place at these higher pH levels.

Differences in the Sedimenting Characteristics of the Cucurbit Seed Globulins

Globulins from the four cucurbit species differ in sedimentation characteristics as the summary in Table II shows. At pH 3.6, squash and pumpkin globulin appear to have associated more rapidly than cucumber and watermelon globulin as Fig. 35G, 37F, 39B, and 40B show. At pH 4.3, differences among the globulins from the different species can best be shown by comparing Fig. 35A, 36F, 38C, and 40A. Squash globulin appeared to have been more rapidly dissociated than was pumpkin globulin during the first half hour after dispersal.

Only dispersions of squash globulin contained a third, more rapidly sedimenting, component below the isoelectric point. While one is tempted to say that cucumber and watermelon globulin dissociated almost completely within one-half hour, there is little direct evidence to support this idea. It can only safely be said that both cucumber and watermelon globulin were dissociated more than squash and pumpkin globulin. The main difference between watermelon and cucumber globulin seems to be that watermelon globulin was more highly dissociated within half an hour after dispersal. At pH 4.7 - 4.8, it may be unwise to make direct comparisons between the results from squash and pumpkin globulin on the one hand and the results from cucumber and watermelon globulin on the other because

TABLE II. Values of pH at Which the Components of Cucurbit Globulins Show Significant Differences

pH	Age of dispersion	Components present in cucurbit globulin															
		Squash				Pumpkin				Cucumber				Watermelon			
3.6	1 - 2 days	Sq^3	Sq^7			Pu^3	Pu^7			Cu^3	Cu^7			Wa^3	Wa^7		
4.3	1/2 hour	Sq^3	Sq^7	Sq^{12}		Pu^3	Pu^7			Cu^3	Cu^7			Wa^3			
4.7 - 4.8	1 day		Sq^7	Sq^{12}			Pu^7*			Cu^3	Cu^7			Wa^3	Wa^7		
9.3	1 day		Sq^7	Sq^{12}			Pu^7	Pu^{12}				Cu^{12}				Wa^{12}	
10.7	1 day		Sq^7	Sq^{12}***			Pu^7	Pu^{12}***			Cu^7?**				Wa^7	Wa^{12}	
11.1	1 day		----				----				Cu^7	Cu^{12}			Wa^7	Wa^{12}	

* Rapidly sedimenting aggregate (or aggregates) was present.

** The behaviour of this preparation is impossible to explain from the information available, so is ignored in the discussion.

*** Found in trace amount only.

the methods of dispersing the two groups differed. As there seems to be a critical point between pH 4.6 and pH 4.8 for squash globulin at pH 4.7, the difference found may be apparent and not real. It does, however, seem probable that differences would have been found using the same methods of dispersal and the same pH. The main difference found among the four species of globulins was: a much higher degree of association in squash and pumpkin globulin than in cucumber and watermelon globulin. Close to the isoelectric point (pH 5.9 - 6.0) the globulins from all four species gave similar sedimentation results. Above the isoelectric point at pH 9.3, there were differences, as shown in Table II. It is quite apparent that there is less tendency for the component of $S^{o}_{20} \simeq 7$ to occur in the globulins from cucumber and watermelon.

Differences below the isoelectric point could be explained by different rates of association or dissociation. Above the isoelectric point the degree of association appears to vary although rates were not studied. The binding sites for association appear to differ among the species of globulin as indicated by different rates of association.

Although each species of globulin differs from the others, squash and pumpkin globulin differ more from cucumber and watermelon globulin than they do from each other. Cucumber and watermelon globulins were more alike than they were like squash and pumpkin globulins. The sedimentation differences among the globulins of the four species correlate better with the published amino acid differences than do the electrophoretic results.

The results show that low pH values, except below pH 2.0, and/or low salt concentrations are accompanied by dissociation of these globulins. On the other hand, high pH values and/or high salt concentrations are accompanied by association.

Danielson (18) was of the opinion that the squash seed globulins should be reinvestigated in higher salt concentrations. This was shown to be of little value below the isoelectric point and of doubtful value above the isoelectric point, as high salt concentrations tend to mask dissociation in this protein.

DISCUSSION

A knowledge of the possible origin and nature of the various components would be of value in explaining the sedimentation results. However, without knowledge of the frictional coefficients of the various components, little can be said of their origin. Since it now appears possible to find the frictional coefficients of certain components by the Archibald procedure (6, 12, 24, 73), any detailed discussion would be premature in view of the fact that only one determination was attempted in the present study. Some implications arising from the sedimentation results should, however, be briefly discussed.

The globulin molecule appears to associate at low pH values in spite of probable molecular rearrangement. Assuming that (a) the molecules were extended or expanded (or both) at low pH values (16), (b) the more rapidly sedimenting component was an associated product of the more slowly sedimenting component, and (c) both the associated and the dissociated molecules change in shape, then the binding sites

for association were not affected enough by the change in shape to prevent association. There is sufficient evidence to believe that the assumptions are probably correct. Although the association process is retarded by low pH values, it occurred, indicating that binding sites were available though perhaps modified. It would be expected from these observations that the binding sites for association are specific areas or even specific points on the protein molecules. This does not exclude the possibility that different types of binding sites are responsible for association at lower pH values than at higher pH values.

There is evidence that the decrease in sedimentation coefficients was reversible. If globulin molecules undergo reversible changes in configuration, the variation in sedimentation coefficients under similar conditions also could be explained.

The experimental sedimentation coefficients were not inconsistent with those calculated for dissociated products even though dissociated products of a dimer would ordinarily be expected to have a sedimentation coefficient higher than one-half the value for the dimer. This would be true only if little or no molecular rearrangement occurred. A prolate ellipsoid is assumed for the shape of the molecule in the following discussion. In fact, however, sedimentation coefficients of the monomer $S^{\circ}_{20} = 3$ were lower than one-half the value for the dimer $S^{\circ}_{20} = 7$. The molecules probably undergo rearrangement below the isoelectric point and this could cause sedimentation values lower than expected. If it is assumed that the component of $S^{\circ}_{20} = 7$ is a trimer and that it split along the major axis, the theoretical values of the monomer do not fit the experimental

values as well as if it is assumed that this component is a dimer of $S^{o}_{20} = 7$. If the component of $S^{o}_{20} = 7$ were a trimer of components of $S^{o}_{20} = 3$, one is forced to assume a split along the the minor axis of a prolate ellipsoid. In any case, it seems unlikely that a trimer would be formed with no trace of a dimer. The calculations for the theoretical sedimentation coefficients were based on an assumed frictional coefficient of 1.2 and an assumed hydration of 30% (35). The suggestion for this mode of attack was obtained from Johnson (35), Johnson and Shooter (42), Alexander and Johnson (4), and Oncley (59).

No complete explanation can be offered for the lack of correlation between electrophoretic results and sedimentation results. On the other hand, a much more satisfactory explanation can be given the sedimentation results than can be offered for those obtained in electrophoretic studies. The two electrophoretic components found at pH 2.3 were always poorly resolved; thus there is no reason to believe that at pH 4.3 they could not have had the same electrophoretic characteristics and thus remain undetected. In many cases age and salt concentration of dispersions used for electrophoresis and for sedimentation differ and thus no rigid comparison can be made. However, electrophoretic and sedimentation experiments at the same salt concentrations seem to be just as difficult to reconcile and thus it is doubtful that the discrepancies can be explained without more extensive analyses.

The results obtained in this study confirm and extend those obtained by Fuerst et al. (23) with squash seed globulin.

They also support the belief that there is greater similarity between the globulins from squash and pumpkin than between these two and those from cucumber and watermelon. All four species yield globulins that are very sensitive to small changes in pH and salt concentration, although the effects of specific conditions were not the same on all four. Many of the individual results cannot be readily explained but all of these proteins appear to belong to very labile association-dissociation systems.

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